ENDOCRINOLOGY

VOLUME 69 AUGUST, 1961 NUMBER 2

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THE INFLUENCE OF OVARIAN HORMONES ON GOITROGENESIS

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ABSTRACT

The influence of ovarian hormones on the pituitary thyroid system was investigated in rats receiving propylthiouracil by observing the effect on the size of the resultant goiter. It was confirmed that estrogen interfered with full goiter development and it was demonstrated that progesterone enchanced goiter formation. When both hormones were combined, the results were the algebraic sum of the individual effects. It is concluded that estrogen and progresterone have opposing effects upon the pituitary-thyroid system.

NO UNANIMOUS concept of the effect of estrogen on thyroid function has emerged and endured, despite considerable investigation. It has been suggested that estrogens influence the thyroid gland in several counterbalancing ways: (a) by inhibition of thyrotrophic (TSH) release from the hypophysis (1), (b) by potentiation of TSH action at the thyroid level (2, 3), (c) by diminution of peripheral utilization of thyroxin (4), and (d) by increasing the thyroid secretion rate (5).

Castrated adrenalectomized male rats given estradiol benzoate in varied doses (10, 20 and 50 μg) for one week, showed similar *in vivo* increases in I^{131} uptake by the thyroid. Although no correlation between dosage and effect could be seen, the overall *in vitro* uptake of I^{131} by the thyroid glands of rats treated for one week with 50 to 300 μg of estradiol benzoate daily, was larger than that of untreated control animals (6). On the other hand, uptake of tracer doses of I^{131} by thyroids of female rats was not influenced by ovariectomy or by administration of ovarian hormones (7). Stoddard *et al.* (8) found no consistent alteration in concentration of serum precipitable iodine either acutely (days) or chronically (months) after castration of young women.

Protein bound iodine levels (PBI) were determined in 3 females in the reproductive period having normal menses and in 2 males on consecutive

Received May 26, 1960.

days for a period of at least one month. In the females during the luteal phase of the cycle, PBI levels were higher and oscillation in values greater than during the follicular phase. "The PBI curves for the two males were similar and the curves for the three females resembled each other; however, there was no similarity between the curves for males and those for females" (9).

When propylthiouracil was used, estrogen caused a reproducible effect upon thyroid size which varied in accordance with dose and physiological state of the animals (10). In such experiments estrogen interfered significantly with the goitrogenic action of propylthiouracil (PTU) in the adult rat.

Since estrogen reduces goiter formation, one might expect progesterone to produce the opposite effect. There are few studies of the influence of progesterone on thyroid activity, viz: Emge reported that "short term" parenteral estrogen (in doses of 200 I.U. ketohydroxyestrin 5 times a week for 10 weeks) given to castrated rats on long term goitrogenic diet had no effect on thyrohyperplasia, although parenteral progesterone (in doses of 0.2 I.U. at same dose and time) caused decreased thyrohyperplasia (11). On the other hand, Meloni (12) studied the effect of progesterone (in 10, 20, 30, and 50 mg. doses) on unstimulated rat thyroid and suggested that progesterone increased thyroid activity. His criteria were histological. Welch (13) showed a significant increase in average follicular size of the thyroid gland in rats given 1 mg. of progesterone for 7 days. When progesterone and 2 mg. of premarin or 2 mg. of premarin alone were given for 7 days, the follicular size decreased to control levels.

The differences in interpretations of the effects of estrogen on the thyroid gland and the paucity of information about the influence of progesterone on the thyroid, prompted us to study the effect of progesterone alone and combined with estrogen, on goitrogenesis in the rat.

MATERIALS AND METHODS

Two successive experiments were carried out using a total of one hundred sixty-two Sprague-Dawley albino virgin rats, with initial weights ranging from 199–227 gm.

Animals were kept under identical conditions except for variations in drug administration. They were given Purina fox chow and tap water *ad libitum*.

Injections of propylthiouracil (PTU) and ovarian hormones[1] were performed daily for 20 days and the animals sacrificed on the day following the last injection. Two mg. of PTU were injected subcutaneously daily, suspended in 0.5 cc. of distilled water with an equal quantity of gum acacia added as dispersing agent. Estradiol benzoate (50 μg) and progesterone (3.5 mg.) were injected subcutaneously daily in 0.1 cc. sesame oil singly or in combination as indicated. All animals received the same volume of distilled water and sesame oil throughout.

[1] We are indebted to Dr. George Babcock Jr., Schering Corporation, for donations of estradiol and progesterone, and to Dr. Stanton M. Hardy, Lederle Division, American Cyanimid Company, for supplying the propylthiouracil.

TABLE 1. EFFECT OF OVARIAN HORMONES ON THYRIOD WEIGHT OF RATS RECEIVING PROPYLTHIOURACIL

Experimental Treatment	No. of Animals	Mean Body Weight	Mean Thyroid Weight/ 100 gm. B.W.
		gms.	mg.
Experiment A			
Sesame oil + water	10	247	4.8
Sesame oil + 2 mg. PTU	20	248	25.0
50μg Estrogen + 2 mg. PTU	20	234	20.8
3.5 mg. Progesterone + 2 mg. PTU	20	241	28.1
50μg Estrogen + 3.5 mg. Progesterone + 2 mg. PTU	20	227	22.2
Experiment B			
Sesame oil + 2 mg. PTU	10	212	21.5
50μg Estrogen + 2 mg. PTU	10	186	16.9
3.5 mg. Progesterone + 2 mg. PTU	10	226	23.7
50μg Estrogen + 3.5 mg. Progesterone + 2 mg. PTU	10	217	19.8

Drug dosages were chosen to provide maximum thyroid weight changes so that relationships between the hormones might be amplified.

At autopsy thyroid glands were dissected clean and weighed to the nearest 0.1 mg. on a torsion balance. The glands were fixed in formalin and examined histologically.

The results were evaluated statistically by analysis of variance (14).

RESULTS

Results obtained are summarized in Table 1. This work confirms that estrogen interferes significantly with full PTU goiter formation (10). Rats given PTU and progesterone show a significant increase in thyroid weight over control PTU animals in Experiment B verifying the borderline results of Experiment A. When progesterone and estrogen are given together the result is roughly the algebraic sum of the individual effects of the two hormones. The probability values are given is Table 2. In both experi-

TABLE II. STATISTICAL EVALUATION OF EXPERIMENTAL DATA
(Thyroid Weights in Mg. per 100 Gm. Body Weight)

Experiment A	No Estrogen (No E)	Estrogen (E)	Difference (E − No E)
No Progesterone (No P)	25.0	20.8	−4.2
Progesterone (P)	28.1	22.2	−5.9
Difference (P − No P)	+ 3.1	+ 1.4	

Within Group S.D. = 5.4 mg. percent.

Estrogen associated with average decline of 5.0 ($P < 0.001$)
Progesterone associated with average increase of 2.2 ($P < 0.1$ but > 0.05)

Experiment B	No Estrogen (No E)	Estrogen (E)	Difference (E − No E)
No Progesterone (No P)	21.5	16.9	−4.6
Progesterone (P)	23.7	19.8	−3.9
Difference (P − No P)	+ 2.2	+ 2.9	

Within Group S.D. = 3.3 mg. percent.

Estrogen associated with average decline of 4.3 (significance $P < 0.001$)
Progesterone associated with average increase of 2.6 ($P < 0.05$)

ments, the decline with estrogen is considerably greater than the increase associated with progesterone in the doses given.

Because of the lower standard deviations in Experiment B as well as the slightly different level of the results obtained in the two experiments, Experiments A and B can not be combined for statistical purposes and are considered separately.

Microscopic examination of the goitrous thyroids of all the experimental groups fails to reveal cytological changes which are distinguishable from those produced by PTU alone. Further histologic studies are in progress.

DISCUSSION

These experiments suggest that estrogen and progesterone have opposing effects upon thyroid function. This antagonism seems to indicate a greater activity for estrogen than progesterone at the dosage levels employed.

Our data indicate progesterone may produce stimulation of the pituitary-thyroid system. This is in keeping with the clinical observation of increased size of the thyroid gland during pregnancy, and in some individuals before each mensis.

The specific character of these interrelations is currently under investigation in patients with thyroid and ovarian disease.

Acknowledgments

The authors wish to express their appreciation to Dr. Hyman Menduke for his kind assistance and advice in preparing the statistical portions of this paper. The assistance of Constance Krolczyk, National Foundation for Infantile Paralysis Medical Student Fellow and Joyce A. McCaughan, Public Health Medical Student Fellow, is gratefully acknowledged.

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EFFECT OF THYROTROPHIN ON THE METABOLISM OF IODIDE^{131} IN THE THYROID GLAND

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ABSTRACT

Iodide^{131} and PBI^{131} in the arterial and thyroid venous blood obtained by catheterization in 28 dogs was measured to study the effect of TSH on the release and uptake of iodide^{131}.

When the glandular organic iodine was labeled by the injection of I^{131} prior to the experiments, release of iodide^{131} by TSH was far more intense than that by KSCN, even if the T/S ratio was decreased to less than 50% of the initial value by the injection of KSCN. On the contrary, the release of iodide^{131} by TSH was only temporary and definitely smaller than that by KSCN when all of the I^{131} in the thyroid was kept in the form of iodide by the administration of methyl mercaptoimidazole.

The ratios of iodide^{131} to PBI^{131} in the thyroid venous output were compared with the ratios of iodotyrosine^{131} to iodothyronine^{131} in the thyroid gland. Whether TSH was given or not, both ratios were equal when methyl mercaptoimidazole was injected to prevent the reutilization of iodide; when methyl mercaptoimidazole was omitted, the latter was greater than the former. The rate of organic binding of iodide^{131} was calculated from the output of iodide^{131} and PBI^{131}, and the ratio of iodotyrosine^{131} to iodothyronine^{131}. It was shown that the organic binding of iodide^{131} was accelerated within a few minutes after the injection of TSH.

From these experiments, it is concluded that in the physiologic state, iodide is always produced from thyroglobulin in the thyroid, but it is not discharged from the gland because the capacity for organic binding of iodide exceeds the iodide production. Both proteolysis of thyroglobulin and organic binding of iodide are accelerated within a few minutes when 50 to 2000 U.S.P. milliunits /dog of TSH is given. However, in the initial phase of its action the rate of iodide production from iodotyrosine exceeds the rate of organic binding of iodide, and this results in the release of iodide from the thyroid gland in addition to PBI. The thyroid gland starts to accumulate iodide several hours later, since the increased proteolysis of thyroglobulin terminates, as judged from the output of PBI, while the increased rate of organic binding continues.

IT HAS been well established that TSH accelerates the release of thyroid hormone and increases the uptake of inorganic iodine by the thyroid gland. While the acceleration of the hormone release takes place within several minutes after the injection of TSH, there is a latent period of

Received August 23, 1960.

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several hours before the thyroid gland starts to increase its uptake of inorganic iodine (1, 2, 3). Recently it has been reported that TSH releases inorganic iodine, in addition to PBI, from the thyroid in the early phase of its action (4, 5, 6, 14).

In order to study the mechanisms of these phenomena, we have attempted to identify the precursors of the released iodide and to establish the time response relationships between the release and uptake of both organic and inorganic iodine.

MATERIALS AND METHODS

Twenty eight male dogs weighing about 10 kg. and maintained on a low iodine diet for two weeks were utilized. In twenty two dogs, 100 to 500 μc. of carrier free I^{131} were injected intramuscularly to label the glandular iodine twenty hours to seven days prior to the experiments. In six dogs (Group II and III) a similar amount of I^{131} was injected two hours after the administration of 200 mg. of methyl mercaptoimidazole, and the experiments were started 20 hours thereafter. Under morphine anesthesia (100 mg.), a midline incision was made in the neck to dissect out the thyroid gland and its vessels. The thyroid venous blood samples were collected from polyethylene tubes inserted into the inferior thyroid veins. A catheter was placed in the femoral artery to collect arterial blood samples and to measure arterial blood pressure. Another catheter was inserted into the femoral vein to infuse heparin and the test substance during the experimental period. Blood transfusion was used if necessary to maintain constant blood pressure. At intervals of 15 to 30 minutes during the control and experimental periods blood samples were collected from the catheters in the thyroid veins and femoral artery, and blood pressure at each collection time was recorded. The blood flow of the thyroid vein was estimated by means of the volume of blood collected during each interval.

Measurement of PBI^{131}

Samples from the thyroid veins and femoral artery were centrifuged to separate 1 ml. of plasma. Radioactivity of the plasma, measured in a well type scintillation counter for 5 minutes, represented the total plasma radioactive iodine. Five ml. of 20% trichloroacetic acid was added in the same tube to precipitate the protein bound iodine, and the precipitate was washed twice with 5 ml. of 10% trichloroacetic acid. The activity of the precipitate redissolved in 0.5 ml. of 2N sodium hydroxide, which was measured in a well type scintillation counter for 5 minutes, represented the PBI^{131} concentration in 1 ml. of plasma. The concentration of inorganic $iodine^{131}$ was expressed as the difference between the total activity and that of the PBI^{131}.

Radiopaperchromatographic technic

About 100 mg. of the thyroid gland was removed and homogenized in 5 ml. of veronal buffer (7), pH 8.6, which contained methyl mercaptoimidazole, 10^{-3}M, in order to prevent the further oxidation of iodide. After boiling homogenates for 5 minutes, 2 mg. each of trypsin and pancreatin were added. Hydrolysis was allowed to proceed at 38° C for 48 to 72 hours. An additional 2 mg. of trypsin and pancreatin were added at 24 hour intervals. Following digestion, 0.1 ml. of homogenate was placed on Toyo No. 50 filter paper in a butanol-acetic acid water (75-10-15) ascending system for 15 to 20 hours. The paperchromatogram was then cut into 0.5 cm. horizontal strips for radioactivity measurement in a well type scintillation counter.

Estimation of the output of PBI^{131} and iodide^{131}.

Output of PBI^{131}

= (concentration of PBI^{131} in the thyroid venous plasma − concentration of PBI^{131} in the femoral arterial plasma) × plasma flow of thyroid vein.

Output of iodide^{131}

= (concentration of iodide^{131} in the thyroid venous plasma − concentration of iodide^{131} in the femoral arterial plasma) × (plasma flow of thyroid vein + red cell flow of thyroid vein × 1.0/1.8)*

RESULTS

Group I (3 experiments)

I^{131} was injected 20 hours before the experiment to label the glandular iodine. Following canulation and after stabilization of blood flow, a control period of 30 minutes was allowed to elapse, then 0.5 gm. of KSCN was administered intravenously over a period of 15 minutes.

Additional KSCN was given in saline at the rate of 0.1 gm. per hour. One USP unit of TSH was injected intravenously 3 hours after KSCN administration, and the effect of TSH was observed for 90 minutes. As shown in Fig. 1A, Table 1, release of iodide^{131} from the thyroid gland was observed immediately after the injection of KSCN, reaching a peak within an hour. When TSH was administered 3 hours after KSCN, the release of iodide^{131} increased intensely, in spite of the decrease in the iodide concentrating mechanism produced by KSCN.

The output of PBI^{131} was not changed by the injection of KSCN.

Group II (3 experiments)

Two hundred mg. of methylmercaptoimidazole was administered intravenously 2 hours before I^{131} injection to prevent the organic binding of I^{131} and an additional 50 mg. of methyl mercaptoimidazole was injected at five-hour intervals. One U.S.P. unit of TSH was injected 20 hours after the I^{131} injection. KSCN was administered 2 or 3 hours after TSH in a manner similar to that used in Group I, to compare the effects of TSH and KSCN.

The release of iodide^{131} initiated by TSH was temporary, lasting only about 90 minutes. It was less intense than that caused by KSCN (Fig. 1 B, Table 1).

* Because iodide is distributed in both plasma and red cells in the ratio, 1.8:1.0, while practically all of the protein bound iodine is in plasma (8, 9).

Experiments were designed with respect to the following questions:

Part I. (Groups I, II, III)

to find whether the release of iodide is due to deiodination or is due to a change in the permeability of cell membranes.

Part II. (Groups IV, V, VI)

to determine the nature of the precursor of the iodide released by TSH, and to study the timeresponse relationships between the release and organic binding of iodide, accelerated by TSH.

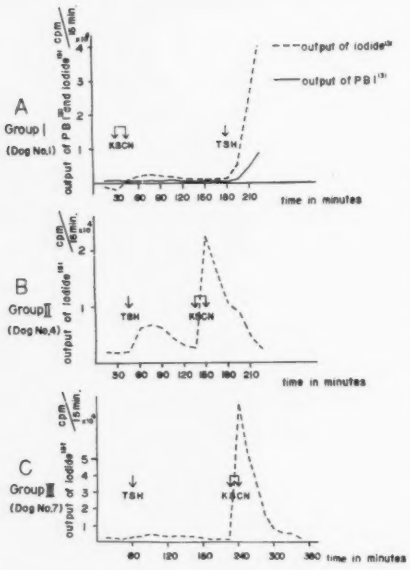

FIG. 1. Representative experiments in Groups I, II, III.

When the glandular organic iodine was labeled by the injection of I^{131} prior to the experiments, release of iodide^{131} after TSH administration was far more intense than that caused by KSCN, even if the T/S ratio was decreased by the injection of KSCN (A. Group I).

On the other hand, the release of iodide^{131} by TSH was only temporary and the total was definitely smaller than that caused by KSCN when all of the I^{131} in the thyroid was kept in the form of iodide by the administration of methyl mercaptoimidazole (B. Group II).

When organic iodine^{127} in the thyroid gland was decreased by antithyroid drug treatment for 3 weeks, the ratios of iodide^{131} release by TSH to that by KSCN became lower than the corresponding values in group II (C. Group III).

TABLE 1. RESULTS OF EXPERIMENTS IN GROUPS I, II AND III

Group NO.	Dog NO.	Time after I^{131} inj. (hours)	Distrib. of I^{131}	Period of	Time to reach max. out put (minutes)	Max. out put of org. iodine (cpm/15 min.)	Max. out put of inorg. iodine (cpm/15 min.)
I	1	20	org. & inorg.	control		590	− 1980
				KSCN	30′	493	2450
				TSH	60′	8700	40000
	2	96	org. & inorg.	control		546	− 749
				KSCN	60′	3070	4910
				TSH	60′	11900	26000
	3	20	org. & inorg.	control		345	− 1520
				KSCN	30′	240	8110
				TSH	60′	1400	19450
II	4	20	inorg.	control			20900(1.0)
				TSH	15′		69800(3.3)
				KSCN	<15′		225000(10.8)
	5	20	inorg.	control			4452(1.0)
				TSH	15′		17410(3.9)
				KSCN	15′		78200(17.5)
	6	20	inorg.	control			315(1.0)
				TSH	30′		360(1.1)
				KSCN	<15′		3980(12.7)
III	7	20	inorg.	control			1297(1.0)
				TSH	30′		2750(2.1)
				KSCN	<15′		57772(44.5)
	8	20	inorg.	control			980(1.0)
				TSH	60′		7630(7.8)
				KSCN	<15′		130000(132.0)
	9	20	inorg.	control			1150(1.0)
				TSH	30′		6400(5.6)
				KSCN	<15′		91200(79.0)

Group III (3 experiments)

Experiments similar to those in Group II were performed, except that the dogs were given 50 mg. of methyl mercaptoimidazole daily for 3 weeks prior to the experiments.

The ratio of iodide^{131} release by TSH to that by KSCN became lower than the corresponding values in Group II. (Fig. 1 C, Table 1).

Doses of KSCN used in these experiments were sufficient to lower the T/S ratio to 30% ±7% (without methyl mercaptoimidazole) and 15% ±3% (with methyl mercaptoimidazole) of the initial values.

Group IV (4 experiments)

Ratios of labeled iodotyrosine to iodothyronine before and after TSH injection were compared in the same thyroid, in both methyl mercaptoimidazole treated and untreated dogs. I^{131} was injected 24 hours before the experiment. Two hours after the administration of 200 mg. of methyl mercaptoimidazole, TSH was injected intravenously. One pole of the thyroid was removed just before TSH injection, and 1.5 hours later, the other pole of the same thyroid was also removed. Both fragments were analyzed by chromatography. The function of the gland was apparently preserved in spite of the injury, as judged by the normal response in the output of I^{131} in the thyroid vein.

As shown in Table 2, the difference between the ratios of labeled iodotyrosine to total I^{131} before and after the injection of TSH was insignificant.

Group V (10 experiments)

I^{131} was injected 20 hours to 5 days before the experiments. The experi-

TABLE 2. RESULTS OF EXPERIMENTS IN GROUP IV

In this group I^{131} was injected 24 hours prior to the experiments. The percent of iodotyrosine was not affected by TSH. The amount of iodide in the gland was less than 10% in all experiments and was not affected by TSH. R: right thyroid, L: left thyroid.

Methyl mercapto imidazole	Dog NO.		Period of	MIT (%)	DIT (%)	T_3 + T_4 (%)	MIT+DIT / Total (%)
treated	1	R	control	60.0	34.5	5.5	94.5
			TSH	61.5	31.5	7.0	93.0
		L	control	62.0	31.5	6.5	93.5
			TSH	60.0	34.5	5.5	94.5
	2	R	control	56.0	30.0	14.0	86.0
			TSH	51.0	34.5	14.5	85.5
		L	control	54.0	30.0	16.0	84.0
			TSH	58.0	28.0	14.0	86.0
untreated	3	R	control	34.0	46.0	20.0	80.0
			TSH	43.5	39.0	17.5	82.5
		L	control	33.0	53.0	14.0	86.0
			TSH	53.5	33.0	13.5	86.5
	4	R	control	47.0	45.0	8.0	92.0
			TSH	28.5	58.0	13.5	86.5
		L	control	42.0	50.5	7.5	92.5
			TSH	36.0	53.5	10.5	89.5

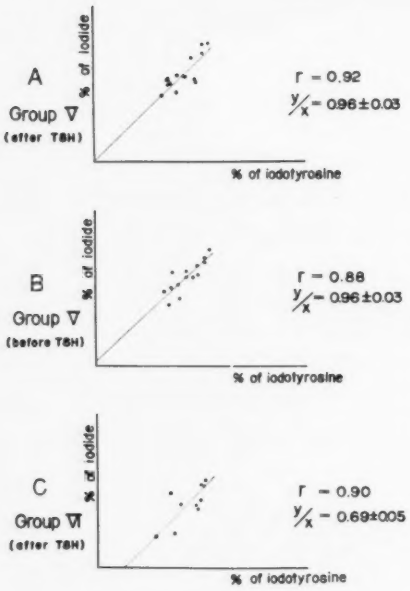

FIG. 2. Correlation between the ratios of iodide^{131} to total I^{131} in the thyroid venous output and the ratios of iodotyrosine^{131} to total I^{131} in the thyroid gland.

Whether TSH was given or not, both ratios were equal when methyl mercaptoimidazole was injected to prevent the reutilization of iodide (A. B.; Group V); when methyl mercaptoimidazole was omitted, the latter was greater than the former (C; Group VI).

ments were started 2 hours after the injection of 200 mg. of methyl mercaptoimidazole to prevent the reutilization of iodide.

After a control period of 30 to 45 minutes, 1 U.S.P. unit of TSH was injected intravenously, and the effect of TSH was observed for 60 to 90 minutes thereafter. Thyroids were then removed, homogenized, hydrolyzed and analyzed by paperchromatography. The ratios of labeled iodotyrosine to iodothyronine in the thyroid glands were compared with the ratios of iodide^{131} to PBI^{131} in the thyroid venous output, before and after the injection of TSH.

During the experimental period after the injection of TSH, the correlation coefficient between iodide^{131}/total I^{131} in the thyroid venous output and iodotyrosine^{131}/total I^{131} in thyroglobulin was 0.92 ($P < 0.01$) and the ratios of both values averaged 0.96 ± 0.03 (Mean $\pm$ S.E.) (Fig. 2A, Table 3). During the control period before the injection of TSH, the correlation coefficient was 0.88 ($P < 0.01$) and the average of the ratios was 0.96 ± 0.03 (Fig. 2B, Table 3). That is, when the thyroid glands were blocked by the injection of methyl mercaptoimidazole to prevent the organic binding of iodide, the ratios of iodide^{131} to PBI^{131} in thyroid venous output were always equal to the ratios of labeled iodotyrosine to iodothyronine in the thyroid gland, whether exogenous TSH was given or not (Fig. 3A).

Group VI (5 experiments)

The procedure used for this group of dogs was similar to that of group V, except that the injection of methyl mercaptoimidazole was omitted.

TABLE 3. RESULTS OF EXPERIMENTS IN GROUP V AND VI

A: Iodotyrosine^{131}/total I^{131} in thyroglobulin (%).
B: Iodide^{131}/total I^{131} in the thyroid venous output before TSH injection (%).
C: Iodide^{131}/total I^{131} in the thyroid venous output after TSH injection (%).

Group NO.	Thyr. NO.	Dog NO.	Time after I^{131} inj. (days)	A (%) MIT+DIT / $+T_3+T_4$ MIT+DIT	iodide / iodide+PBI B (%) before TSH	C (%) after TSH	B / A	C / A	B / C
▽	1	1 R	1	63		69		1.10	
	2	L	1	63		63		1.00	
	3	2 R	2	70	68	57	0.97	0.81	1.20
	4	3 R	1	83	74	86	0.89	1.04	0.86
	5	4 R	1	87	76	65	0.87	0.75	1.17
B/A	6	5 R	20h	98	97	98	0.99	1.00	0.99
=0.96±0.003	7	6 R	1	93	90	97	0.97	1.04	1.00
	8	L	1	93	86	90	0.93	0.97	0.89
C/A	9	7 R	2	86	84	68	0.98	0.79	1.24
=0.96±0.03	10	L	2	77	79	70	1.02	0.91	1.13
	11	8 R	5	71	56	71	0.79	1.00	0.79
B/C	12	L	5	62	51	67	0.82	1.08	0.76
=1.03±0.04	13	9 R	5	65	78	64	1.20	0.99	1.22
	14	L	5	58	62	54	1.07	0.93	1.15
	15	10 R	5	64	65	66	1.01	1.03	0.99
	16	L	5	77	74	70	0.96	0.91	1.06
▽I	17	11 R	1	92	neg	74	neg	0.74	neg
	18	L	1	91	neg	69	neg	0.76	neg
	19	12 R	1	66	neg	29	neg	0.44	neg
	20	L	1	50	neg	27	neg	0.54	neg
	21	13 R	5	84	neg	53	neg	0.66	neg
C/A	22	L	5	86	neg	50	neg	0.58	neg
=0.69±0.05	23	14 R	2	88	neg	70	neg	0.79	neg
	24	L	2	88	neg	57	neg	0.65	neg
	25	15 R	1	62	neg	63	neg	1.01	neg
	26	L	1	71	neg	54	neg	0.76	neg

FIG. 3

A. Representative experiment in group V (Dog No. 10). Note the ratios of iodide^{131} to PBI^{131} were always constant.
B. Representative experiment in Group VI. (Dog. No. 13). The rate of organic binding was accelerated by TSH within a few minutes.

———— output of PBI^{131}.
- - - - - - - - output of iodide^{131}.
— — — — — — calculated production of iodide.^{131}
═══ amount of iodide^{131} bound organically.

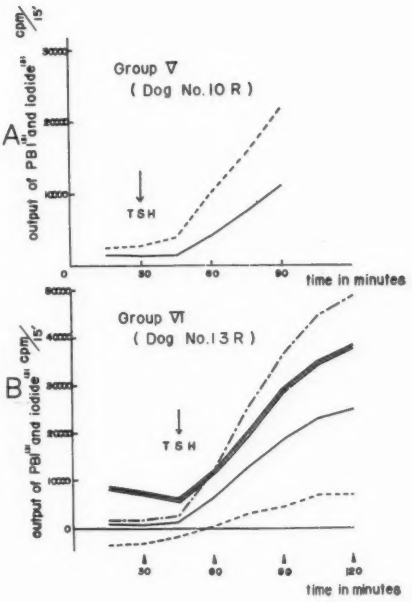

TABLE 4. CHANGE OF ORGANIC BINDING OF I^{131} BY TSH

Note that the organic binding was accelerated by TSH. The period of observation of TSH effect in this group is from 45 to 60 minutes after the injection of TSH.

Dog No.		Period of	MIT+DIT / Total (%)	Output of PBI^{131} ($\frac{cpm}{15'}$)	Calculated Production of iodide^{131} ($\frac{cpm}{15'}$)	Measured Output of iodide^{131} ($\frac{cpm}{15'}$)	Amount of iodide^{131} bound org. ($\frac{cpm}{15'}$)
11	R	control		5	57	−1310	1367
		T S H	92	2555	29400	3970	25430
	L	control		79	800	−1980	2780
		T S H	91	3620	36600	3670	32930
12	R	control		900	1746	−6740	8486
		T S H	66	25050	48700	10500	38200
	L	control		600	600	−3990	4590
		T S H	50	25200	25200	7440	17760
13	R	control		128	672	0	672
		T S H	84	9400	49400	17200	32200
	L	control		140	860	110	750
		T S H	86	1834	31500	5070	26430
14	R	control		209	1532	−428	1960
		T S H	88	525	3850	1340	2510
	L	control		210	1540	−1700	3240
		T S H	88	14400	105800	22550	83250
15	R	control		148	242	−1035	1277
		T S H	62	800	1305	−937	2242
	L	control		38	93	−1530	1623
		T S H	71	551	1350	−1830	3180

In this group of animals, the ratios of iodide^{131}/total I^{131} to iodotyrosine^{131}/total I^{131} averaged 0.69 ± 0.05 (Mean $\pm$ S.E.), indicating that a certain fraction of the iodide^{131} produced in the thyroid was reutilized and bound organically. In order to express the rate of organic binding of iodide^{131}, the following formula was used:

Amount of iodide^{131} bound organically
= calculated amount of iodide^{131} produced in the gland − measured output of iodide^{131} from the gland.

Calculated amount of iodide^{131} produced in the gland

$$= \text{output of PBI}^{131} \times \frac{\%\text{ of iodotyrosine}^{131}\text{ in thyroglobulin}}{\%\text{ of iodothyronine}^{131}\text{ in thyroglobulin}}$$

As shown in Fig. 3B and Table 4, the rate of organic binding was accelerated by the injection of TSH apparently within several minutes.

DISCUSSION

It has been reported recently that TSH releases inorganic iodine, in addition to PBI, from the thyroid in the early phase of its action (5, 6). It is still undecided, however, whether the release of inorganic iodine is due to deiodination of some products of the increase in thyroglobulin proteolysis, or if it is due to a change in the permeability of the epithelial cell membranes (5). The nature of the precursor of the iodide discharged into thyroidal venous blood in response to the administration of TSH is also yet unknown (6).

In our experiments, when the glandular organic iodine was labeled by

prior injection of I^{131}, the release of iodide^{131} by TSH was far more intense than that elicited by KSCN, this was true even when the T/S ratio was first decreased to less than 50% of the initial value by previous injection of KSCN. However, the release of iodide^{131} by TSH was only temporary and the total was definitely smaller than that by KSCN if all of the I^{131} in the thyroid was kept in the form of iodide by the administration of methyl mercaptoimidazole. This temporary release of iodide^{131} by TSH seems to be due to the production of iodide^{127} from the organic iodine ^{127} by the administration of TSH, since this release of iodide^{131} became much smaller when the amount of iodide^{127} produced in response to TSH was decreased by previous methyl mercaptoimidazole treatment for 3 weeks.

From these experiments it is concluded that the release of inorganic iodine in response to TSH is due to production of iodide from organic iodine.

As for the precursor of iodide released by TSH,

1) The accelerating effect of TSH on the deiodination of free iodotyrosine (6, 10) would not give a sufficient explanation of the release of iodide, because the amount of organic iodine not combined with thyroglobulin was smaller than the amount of iodide in the thyroid gland, and the release of iodide by TSH was more intense than that by KSCN as shown in Group I experiments.

2) Thyroid hormone was not deiodinated in the thyroid (11).

It was, then, suspected that the release of iodide by TSH was due to deiodination of iodotyrosine derived from proteolysis of thyroglobulin (12).

To test this hypothesis, after the injection of TSH, the ratios of iodide^{131} to PBI^{131} in the thyroid venous output and the ratios of labeled iodotyrosine to iodothyronine in thyroglobulin were compared. It would be expected, if the deiodinated iodotyrosine were the source of the iodide released from the thyroid gland, that the ratios of iodide^{131} to PBI^{131} should vary in strict parallelism with the ratios of labeled iodotyrosine to iodothyronine. Both ratios were always the same.

The ratio of iodide^{131}/total I^{131} and iodotyrosine^{131}/total I^{131} were similar in the control period if only methyl mercaptoimidazole was injected. These ratios were lower than 1.0 in both the control and experimental periods if methyl mercaptoimidazole was omitted, as shown in Group VI.

The fact that the average of these ratios in the treated dogs (Group V) was slightly below 1.0, might be due to an incomplete effect of methyl mercaptoimidozole in blocking the organic binding.

In these experiments, only I^{131} was measured and the specific activity was not measured. Assuming that radioactive iodine and stable iodine behave in the same way in the thyroid gland, it may be concluded that, in the normal physiologic state, iodide is always produced from thyroglobulin in

the thyroid in addition to PBI, but not discharged from the gland, because the capacity for organic binding of iodide exceeds the rate of iodide production (intrathyroidal iodide cycle) (13). But once 50 to 2000 U.S.P. milliunits/dog of TSH is given, the iodide production exceeds the organic binding and a net iodide discharge from the thyroid gland occurs.

It is well known that there is a latent period of several hours before the thyroid gland starts to increase its uptake of inorganic iodine, while the acceleration of hormone release takes place within several minutes after the injection of TSH (1, 2, 3). Our results, however, should raise the question as to whether this delayed acceleration of iodide uptake is due actually to the delayed acceleration of organic binding or to the reutilization of iodide produced from thyroglobulin, masking the accelerated organic binding of iodide. To explore this question, the rate of organic binding of iodide was calculated according to the results of group V experiments. The organic binding was apparently accelerated by TSH within several minutes, as shown in Group VI.

From our experiments, it is concluded that TSH accelerates both proteolysis of thyroglobulin and organic binding of iodide within several minutes. The observed net release of iodide, in addition to PBI, from the thyroid gland in the initial phase of TSH action, is explicable if the rate of iodide production from iodotyrosine derived from hydrolyzed thyroglobulin exceeds the rate of organic binding. The thyroid gland is observed to start to accumulate iodide several hours later. This may be explained on the basis of the termination of the increased proteolysis of thyroglobulin, while the increased rate of organic binding continues.

Acknowledgments

This investigation was supported by a research grant from the ministry of education of Japan. We are indebted to Miss I. Nurino, and Miss M. Handa for their technical and secretarial service. The scintillation counter was purchased by a grant from the Rockefeller Foundation. TSH used in this study was kindly supplied by the Armour Pharmaceutical Co. and methyl mercaptoimidazole by the Chugai Pharmaceutical Co. We wish to express our thanks to Dr. Sheila B. Blume for correcting the English of the manuscript.

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EFFECT OF PERCUTANEOUS APPLICATION OF TESTOSTERONE PROPIONATE AND OF ESTRADIOL BENZOATE ON THE TOTAL ACID MUCOPOLYSACCHARIDES OF RAT SKIN

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ABSTRACT

The effect of percutaneous application of oil solutions of testosterone propionate and estradiol benzoate on the levels of total acid mucopolysaccharides were examined in rat skin.

After 7 days of treatment, testosterone propionate produced a significant increase in the level of uronic acid ($0.025 < p < 0.05$) and a slight increase of hexosamine ($0.05 < p < 0.1$) by comparison with homologous parts of skin of control oil treated rats. When treatment lasted 14 days a slight increase in the concentration of uronic acid and not of hexosamine was noted. A tendency of estradiol benzoate to cause a decrease of uronic acid concentration in rats treated for 7 days was observed while 14 days treatment did not affect the levels of either constituent. The results seem to agree with the effects of sex hormones on cocks' comb.

It is suggested that the changes in the levels of the sex hormones in the organism rather than their actual levels are responsible for the effects obtained.

IN RECENT years considerable investigation has been carried out on acid mucopolysaccharides in connective tissues and on the changes in their metabolism caused by various external factors. There have been several reports on the possible mechanism involved in the changes in acid mucopolysaccharides metabolism in connective tissue after treatment with adrenocortical hormones. Less information is available concerning the action of sex hormones in this respect.

The effect of testosterone administration on comb growth in cocks is well known. Ludwig and Boas (1) found that growth, differentiation and mucopolysaccharide content of the comb were dependent on androgen stimulation and they identified hyaluronic acid as a major mucopolysaccharide component of its intercellular ground substance. Similar results were obtained on capons by Ber and Mikolajczyk (2) who injected testosterone under the skin at the base of the comb and observed a parallelism between the growth of the comb surface and the amount of mucopolysaccharides determined by a modified colorimetric method with sheep hemoglobin. Szirmai (3) found that the increase of the metachromasia in

Received September 27, 1960.

the connective tissue of the comb coincided with the increase of the total hexosamine content during the development of this organ in the normal rooster as well as during testosterone treatment of the capon. The ratio hexosamine/hydroxyproline showed an increase in both cases and a similar increase was also found in the wattles and to a lesser degree in the earlobes.

On the other hand, estradiol applied locally to immature male chicks prevented normal growth of the comb and, on histological examination, a diminution of the metachromatic interstitial ground substance was revealed (4). The substance however, which accumulates in the sex skin of monkeys during the estrous phase of the menstrual cycle is believed to be hyaluronic acid or a closely related compound (5). Koch (6) obtained similar results in cows. He found that under the influence of synthetic estrogens (Dienestrol and Stilbestrol) a loosening of the connective tissue with water and mucopolysaccharide accumulation took place especially in the neighbourhood of the sex organs.

The influence of the same hormone may also vary in different species, as seen indirectly from the works of Sobel (7), for instance, who found a decreased content of hexosamine in rat skin after α-estradiol administration and of Schmidt (8) who, on subcutaneous injections of pharmacological doses of estradiol benzoate, obtained a rise in the total amount of hexosamine in the skin of mice. The lack of uniformity of responsiveness is observed even within the same species. Selye (9), for example, found that the skin of normal shaved mice did not respond with edema formation to estradiol treatment, in contrast to the hairless strain which manifested a generalized non-pitting cutaneous edema.

Our present study was designed to determine whether Testosterone Propionate and Estradiol Benzoate, applied percutaneously, were able to affect parts of the skin not known to be specifically influenced by sex hormones.

Ber (10), working with capons and smearing the external surface of one wattle with oil solution of testosterone propionate, observed a considerable enlargement of the wattle treated, a smaller enlargement of the second wattle, and, proportionally an even smaller enlargement of the comb. This shows that, in addition to the systemic action produced through the blood circulation, hormones may cause changes in parts of the skin directly treated.

The purpose of our work was to determine whether changes could be obtained in the side of the dorsal skin treated with sex hormones compared to the symmetrical side treated with the solvent alone.

EXPERIMENTAL PROCEDURE AND METHODS

Thirty-five male albino rats from a closed colony grown in our institute and maintained by line-breeding, weighing from 170 to 200 gm. each were used. They were kept in individual cages with free access to water and purina chow. On the first day they

TABLE 1. DIVISION OF RATS UNDER EXPERIMENT ACCORDING TO KIND OF TREATMENT AND ITS DURATION

No. of rats	Group	Duration of treatment in days	Treatment	
			Left side of dorsal skin	Right side of dorsal skin
5	I	7	Testosterone propionate*	Oleum olivarum
5		14	Testosterone propionate*	Oleum olivarum
5	II	7	Estradiol benzoate†	Oleum olivarum
5		14	Estradiol benzoate†	Oleum olivarum
5	III	7	Oleum Olivarum	—
5		14	Oleum Olivarum	—
5	IV	—	—	—

* 2% solution of Testosterone propionate in oleum olivarum.
† 0.5% solution of Estradiol benzoate in oleum olivarum.

were weighed and their dorsal skin shaved with an electric clipper, a thin line of hair being left between the two symmetrical parts of the back. The 35 rats were divided into four groups according to treatment, and each group was subdivided according to duration of treatment into 2 subgroups, as shown in Table 1. The treatment was given every morning and consisted of percutaneous application of 2–3 drops of the substance with a soft brush.

The day following the last application the animals were weighed and killed with chloroform. The dorsal skin was removed and any adherent muscle and fat tissue carefully separated. It was immediately stretched on a corkboard, reshaved with an electric clipper and divided into two parts representing the two halves of the back differently treated. Each half was weighed immediately for comparison with the weight of dry defatted tissue to be obtained later. The portions were then placed into pure acetone for 48 hours (the acetone being changed after 24 hours) and shaken occasionally. They were then cut into pieces of approximately 2×2 mm. and placed in a Soxhlet extraction apparatus with petroleum ether for 4 hours. The skin particles were successively placed in a vacuum desiccator and left overnight above P_2O_5 *in vacuo*. They were then weighed and ground to a fine powder in an electric grinder. The powder was stored in a desiccator above P_2O_5 *in vacuo* up to the time of further processing.

The acid mucopolysaccharide content was estimated by determination of its uronic acid and hexosamine constituents. The effect of incubation with testicular hyaluronidase was also examined in the samples.

The procedure generally used throughout this experiment was based on that described by A. Bollet (11), tested in our laboratory for its reproducibility and recovery of test material with satisfactory results. The method was generally adopted with minor modifications as follows: The samples of dry defatted tissue powder ranging from 0.6–0.8 gm. were brought to solution by stirring for 45 minutes with 40 ml. of 0.5 N NaOH with a mechanical stirrer. No visible material remained in suspension after this procedure. 1.6 ml. of 8.75 M acetic acid were added and then 2.4 ml. perchloric acid 70% for deproteinization. The mixture was refrigerated at 4° C for 1 hour and brought to 55 ml. with cold perchloric acid 4%. 50 ml. of the filtrate were transferred into a cellophane bag for dialysis overnight against running tap water. The content of the bag was then quantitatively transferred into a 100 ml. volumetric flask and brought to the mark with distilled water. From this solution the following aliquots were drawn:

1) Duplicate 10 ml. aliquots for uronic acid determination using the carbazole reaction. 2) A 40 ml. aliquot for hexosamine determination. 3) Duplicate 10 ml. aliquots for the test of hyaluronidase incubation. The procedure involving the precipitation of the acid mucopolysaccharides by protamine sulfate was followed in the subsequent steps of isolation.

For the determination of uronic acid in the protamine sulfate precipitate the carbazole method of Dische (12) was used. For the hexosamine estimation the lyophilized protamine sulfate precipitate obtained from the 40 ml. aliquot of the dialyzed extract was dissolved in 1.5 ml. of 4N HCl and 1 ml. hydrolysed in a glass-stoppered 5 ml. volumetric flask in a boiling water bath for 8 hours. Using Brom Thymol Blue as indicator (13), the hydrolysate was neutralised first with NaOH 2N and afterwards with NaOH 0.25N to the end point. The volume was brought to 5 ml. and 1 ml. aliquots were used for determination of hexosamine according to the method of Elson and Morgan (14). NaCl solution was added to the standard and blank solutions in order to obtain the same level of this salt as in the neutralised sample solutions (15).

RESULTS AND DISCUSSION

The mean values of the concentrations of acid mucopolysaccharides constituents in the homologous parts of the skin for each subgroup as a whole and their standard errors are present in Table 2. The effect of incubation with testicular hyaluronidase is presented as uronic acid concentration

TABLE 2. EFFECT OF PERCUTANEOUS APPLICATION OF TESTOSTERONE PROPIONATE, ESTRADIOL BENZOATE AND OLEUM OLIVARUM ON CONCENTRATION OF CONSTITUENTS OF TOTAL ACID MUCOPOLYSACCHARIDES OF RAT SKIN

Treatment	Group	Duration of treatment in days	Side of dorsal skin	Uronic acid (carbazole reaction) μg/100 mg. dry defatted tissue*	Uronic acid (carbazole reaction) after incubation with testicular hyaluronidase μg/100 mg. dry defatted tissue**	Hexosamine μg/100 mg. defatted tissue*
Testosterone propionate on the left side (L) Oleum olivarum on the right side (R)	I	7	L	119.7±5.2†	42.3±2.8	133.3± 7.6‡
			R	110.2±3.8	41.0±2.0	123.9± 5.9
		14	L	122.6±5.6	42.6±2.0	124.0± 5.9
			R	115.6±3.8	44.7±1.4	120.3±10.6
Estradiol benzoate on the left side (L) Oleum olivarum on the right side (R)	II	7	L	96.9±2.6	44.7±6.8	120.0±10.6
			R	94.6±2.4	41.2±3.3	113.6± 2.5
		14	L	107.6±2.0	42.1±1.4	108.6±10.2
			R	109.7±2.7	41.0±1.3	105.6± 6.2
Oleum olivarum on the left side (L) Right side (R) untreated	III	7	L	102.7±1.6	39.7±3.2	107.4± 7.5
			R	98.2±3.0	42.6±1.0	111.5± 6.2
		14	L	108.3±4.5	43.6±1.7	114.9± 8.1
			R	106.3±5.1	42.5±2.9	119.1± 8.4
Both sides (L, R) untreated	IV		L	107.2±6.5	37.8±2.6	118.2± 2.1
			R	104.2±3.4	37.5±2.5	111.5± 3.1

* Mean values ± their standard errors for each subgroup consisting of 5 male albino rats weighing 170–200 gr.
** Mean values ± their standard errors for each subgroup consisting of 4 male albino rats weighing 170–200 gr.
Formula for standard error:

$$\frac{Sd}{\sqrt{n-1}}$$

† $0.025 < p < 0.05$ in comparison with the corresponding subgroup of group III.
‡ $0.05 < p < 0.1$ in comparison with the corresponding subgroup of group III.
Formula for "t":

$$\frac{X_1 - X_2}{\left[\frac{N_1 + N_2}{N_1 + N_2 - 2}\right]^{1/2} \cdot \left[\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 N_2}\right]^{1/2}}$$

in the residue of the total acid mucopolysaccharides after hyaluronidase treatment.

To examine the possibility of local effect of the two hormones the mean values of mucopolysaccharide constituents found in the two symmetrical dorsal parts of the skin within the same subgroup were compared statistically. The fact that there was no significant difference between the mean values found in the two halves of the dorsal skin of untreated animals (Group IV) provided the basis for such an investigation.

In no group was any significant difference observed between the left and right halves of the dorsal skin and this uniformity of result in addition to the observations to be reported here later, according to which some differences were found between hormone treated and their respective control subgroups, speak for a rather generalized action of these hormones on the skin under the conditions of the experiment described. In the course of the treatment relatively high doses of the applied substances were apparently adsorbed through the skin and entered the circulation. This is supported, f.i. by the observed loss of weight of the estradiol treated group. Decreases in weight ranged from 11 to 32 gm. after 7 days and from 36 to 52 gm. after 14 days, while rats treated with testosterone propionate or oleum olivarum increased in weight from 8 to 50 gm.

The rats belonging to this group consumed during four days 20–40 gm. of food, while, in the same period, control animals (Group III) and the testosterone treated group consumed 51–60 gm. and 40–70 gm. respectively.

The influence of estrogen administration to several species of animals with respect to changes in body weight was studied inter alia by Koch (6). In contrast to other species rats did not respond by weight increase. In female rats, where partial hepatectomy had been performed, Ber (16) found that after injections of diethylstilbestrol there was a decrease in body weight by comparison to that of the untreated or testosterone or oil treated animals similarly operated upon. Brobeck *et al.* (17) found that, in female rats during estrus, appetite is decreased and motility increased, resulting in loss of body weight. In diestrus the opposite was observed. The authors expressed the view that gonadal hormones may exercise some influence on the hypothalamus provoking the changes mentioned.

Our results show that estradiol causes a decrease in weight probably due to decreased appetite.

From the foregoing it may be supposed that sufficiently high quantities of the hormones were adsorbed through the skin to cause action of a general character. As a result, under the conditions of our experiment, eventual changes in acid mucopolysaccharides content in skin could not be observed as a local effect. In order to examine this possibility a second experiment covering a shorter treatment period and with smaller doses is under way.

We were able, however, to show differences in levels of total acid muco-

polysaccharides content when comparing hormonally treated skin parts to the homologous parts of control subgroups (table 2).

Comparison between the corresponding subgroups of Groups III (left side treated with oleum olivarum) and IV (left side of untreated animals) shows that treatment consisting in shaving and application of oleum olivarum for 7 or 14 days did not produce significant differences in the levels of the constitutents examined.

The concentration of uronic acid in the skin of the rats treated with testosterone for 7 days is significantly higher ($0.025 < p < 0.05$) when compared with the control oil treated subgroups. The hexosamine content showed a slight increase ($0.05 < p < 0.1$) in the same subgroup. The longer application of testosterone (14 days) tended to lower the effects since no significant differences were found.

Our results show that testosterone is able to influence the acid mucopolysaccharide content of the dorsal skin of the male albino rat in a similar fashion as seen in the comb of cocks and provides evidence of the general action of this hormone. These findings are not in accordance with the results obtained by Sobel *et al.* (7) who, using different methods and mode of calculation, did not find difference in the content of hexosamine in skin of rats injected with testosterone. These authors determined total hexosamine in skin of young female rats and calculated their findings with regard to body surface and body weight.

Concerning the differences between respective subgroups of Groups II (estradiol treated) and III (oleum olivarum) a tendency to cause a decrease of uronic acid content was observed in rats treated for 7 days. Here also, the longer duration of treatment tends to level out the differences.

The mode of the influence of estradiol seems to agree with that observed on cocks comb (4). Hvidberg and Schou (18), working on subcutaneous absorption of different substances by different techniques in different animals, could not arrive at clear cut conclusions on the changes observed. The concentration of sulphacetamide in blood after subcutaneous injection of this substance in rats and rabbits was unaffected by previous treatment with estrogenic hormone. On the other hand Schmidt (8) was able to demonstrate higher levels of hexosamine in skin of mice after subcutaneous injection of estradiol benzoate. Koch (6) also found an increase in "mucoid intercellular substances" under estrogen influence.

Lack of uniformity in experimental conditions and methods of investigation may be held responsible for such want of agreement.

Our impression is, that the changes themselves in the levels of androgens in the organism seem to be more important than the actual level, as the effect is more pronounced after 7 days treatment than after 14 days. This problem is now under detailed study.

Concentration, nature and degree of polymerization of acid mucopoly-

saccharides, are major factors in the state of hydration of tissues. An attempt was made to correlate the changes observed in the levels of total acid mucopolysaccharides and the percentage of dry defatted tissue in skin of the rats. Comparison of the figures obtained with respect to the differences between heterologous parts of the skin within the same subgroup as well as between homologous parts in subgroups, comparing hormone treated to control subgroups, showed no significant correlation. This could be explained by the plurality of factors involved which are known to influence the water content of the skin.

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RESPONSE OF THE PINEAL AND ADRENAL GLANDS TO SODIUM RESTRICTION[1]

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ABSTRACT

Pineal fat stainable with oil red 0 was markedly depleted in intact and adrenalectomized rats maintained on a diet deficient in sodium. Pineal fat was mildly depleted by adrenalectomy alone. The zona glomerulosa of the adrenal gland widened in response to the sodium deficient regimen in both intact and pinealectomized rats. These alterations were taken to indicate that the pineal gland may elaborate a hormone which is involved in salt regulation.

THE work of Deane, Shaw and Greep (1) and of Hartroft and Eisenstein (2) have established that the zona glomerulosa of the adrenal gland responds to dietary sodium restriction by undergoing morphological changes which have been interpreted by them to indicate augmented secretory activity of this zone. Since these changes also occurred in hypophysectomized animals, it was concluded that the secretion of mineral corticoids by the zona glomerulosa was largely independent of pituitary control (1).

More recently, Farrell (3) has postulated that the secretions from the zona glomerulosa may be controlled by a hormone originating in the pineal-posterior commissure area. He later observed (4, 5) that administration of fatty extracts of pineal glands to dogs resulted in augmented secretion of aldosterone in adrenal venous blood. These works led him to postulate that an "adrenoglomerulotropic" factor was elaborated in this area of the brain stem.

Experiments reported here were designed to explore the relationships between the pineal gland and the zona glomerulosa of the adrenal cortex using a morphological approach.

METHODS AND MATERIALS

Male rats of the Holtzman strain were employed in this study. They were placed on purified diets deficient in sodium or with complete salts. Distilled and tap water were given for drinking purposes. The animals were placed in clean cages to avoid contamination with sodium.

Received October 3, 1960.

[1] Supported by a grant from Los Angeles County Heart Assoc. #271.

Composition of the diet

Anhydrous dextrose	58%
Vitamin-free casein	22%
Wesson oil	12%
Vitamin mixture*	3%
Salts	5%

* Vitamin fortification mixture from the Nutritional Biochemical Co., Cleveland, Ohio.

Composition of the salt mixture

NaCl*	15 gm.
$CaCO_3$	24 gm.
$CaHPO_4$	15 gm.
$FeSO_47H_2O$	3 gm.
K_2HPO_4	35 gm.
$MgSO_4$	7 gm.
Trace mixture	1 gm.

* Replaced with dextose in sodium deficient diet. Composition of the trace mixture was as follows: $MnSO_4$, 50 gm.; KI, 10 gm.; $CuSO_4$, 12 gm.; $ZnCl_2$, 20 gm.; $Co(Ch_3COO)_2$, 0.1 mg

Young adult rats weighing between 100 and 125 gm. at the onset of the experiment were chosen. They were placed on the dietary regimen at the age of 40 days. Intact, adrenalectomized, pinealectomized and incompletely operated animals were used. The adrenalectomized animals were allowed 10 days and the pinealectomized and incompletely operated animals were allowed 5–7 days to recover before being placed on the experimental diets. Since the adrenalectomized group survived the sodium deficient regimen poorly, they were autopsied after one week on the diet. The pinealectomized and incompletely operated rats were autopsied at weekly intervals.

The pineal gland in the rat is located immediately ventral to the confluens sinus where it sometimes adheres to the dura. It is connected to the brain by a long slender stalk. Because of its superficial location, it was possible to devise an operative approach through the cranial vault. After an appropriate skin incision, the connective tissue was cleared away from the calvarium. A trephine 2 mm. in diameter was used to remove a piece of the calvarium immediately lateral to the sagittal suture and anterior to the posterior transverse suture. The dura was then incised and a capillar tube, of appropriate dimension, attached to a suction hose, was directed under the dura toward the location of the pineal gland. The pineal gland was then removed using mouth suction. In this procedure, the pineal gland is not visualized but it is possible to judge from the location of the sutures where it lies. After removal of the gland, the excised bone was replaced and dental resin was applied to seal it back in place. Completeness of the operation was checked at autopsy. This procedure resulted in about 98% recovery and about 65% complete removals of the pineal glands. The animals in which the pineal gland was not removed were considered as sham-operated control animals.

At autopsy, the adrenals, pituitary and pineal glands were removed and weighed on a torsion balance. Differences between the average weights of the glands were statistically compared, using Fisher's method (6). The tissues were fixed in 10% neutral formalin, section at 5–10 μ and stained with oil red O and hematoxylin.

RESULTS

Intact rats fed the purified control diet grew normally and upon autopsy no pathological changes were found. Table 1 lists the body weights and weights of the glands studied.

The weight and histological appearance of the adrenal gland were not

TABLE 1. ORGAN WEIGHTS CALCULATED/100 GM. BODY WEIGHT

Type animal	Diet	B.W.	No. of rats	Paired adrenals	Pituitary	Pineal
Intact	control 1 week	125	8	24.0 ±3.36	4.21± .75	1.36± .28
Intact	control 3 weeks	171	9	15.38± .77	3.64± .75	1.00± .20
Intact	sodium low 1 week	136	9	23.72±2.78	4.01± .51	.75± .34
Intact	sodium low 3 weeks	163	10	22.21± .86	3.37± .85	.56± .31
Adrenalectomized	control 1 week	127	7	—	4.01±1.29	1.06± .32
Adrenalectomized	sodium low 1 week	146	6	—	4.20±1.24	.68± .28
Pinealectomized	control 1 week	184	6	23.20±1.85	3.40±1.00	—
Pinealectomized	control 3 weeks	178	6	21.33±3.08	3.70± .94	—
Pinealectomized	sodium low 1 week	160	6	23.30±1.43	3.40±1.85	—
Pinealectomized	sodium low 3 weeks	174	6	22.41±3.97	4.12±1.31	—
Sham operated	control 3 weeks	198	5	19.55±2.82	3.13±1.00	—
Sham operated	sodium low 3 weeks	173	5	20.99±1.20	2.95±1.07	—

Mean values ±standard error.
Body weight at termination of experiment in gm. Organ weights in mg.

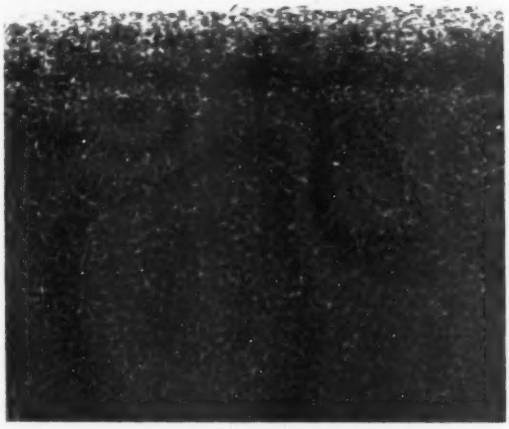

FIG. 1. Adrenal gland taken 3 weeks after pinealectomy and appears normal. ×125.

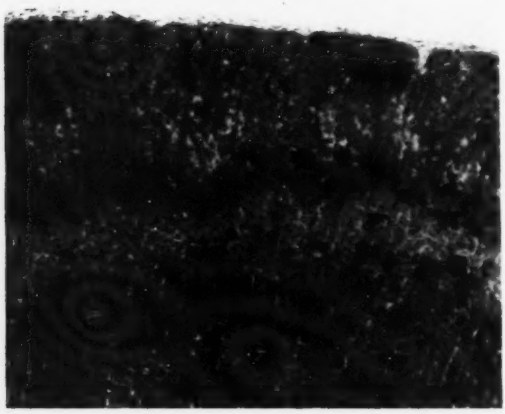

FIG. 2. Adrenal gland was taken from a pinealectomized rat after 3 weeks on a sodium deficient diet. Widening of the glomerulosa and fatty changes are typical of those occurring in intact rats under similar conditions. ×125.

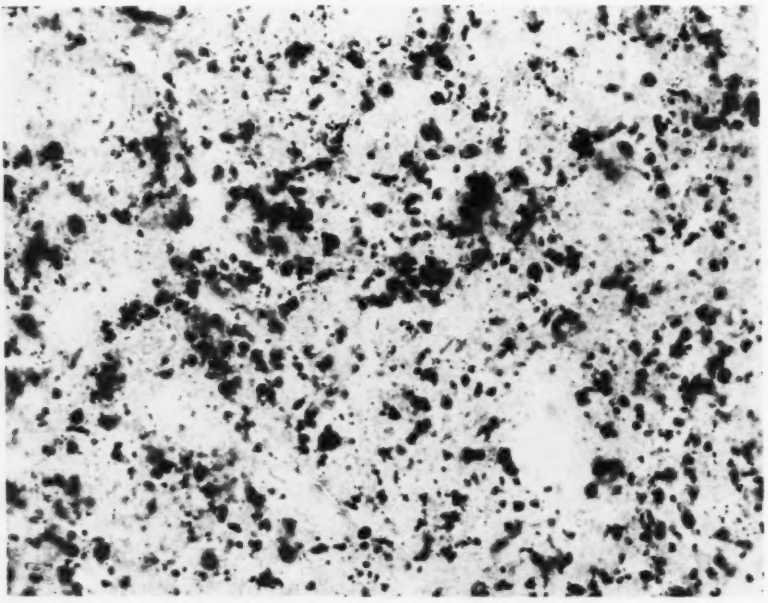

FIG. 3. Pineal gland taken from an intact rat on the control diet and demonstrates the normal distribution of fat. ×500.

altered by removal of the pineal gland in rats fed the control diet. On the other hand, the zona glomerulosa of the pinealectomized rats responded to the low sodium regimen in the same degree and manner as did that of the intact rats (Figs. 1 and 2). The zona glomerulosa was considerably wider in both groups, the cells were hypertrophied, large watery vacuoles were present, and the fat content was less prominent. This characteristic response of the adrenal glomerulosa was similar to that reported by Deane, Shaw and Greep (1) and by Hartroft and Eisenstein (2) under similar dietary conditions.

While the weight of the pineal gland was not altered by removal of the adrenal glands, the amount of fat as seen histologically in the pineal gland was reduced as compared with the intact controls (Figs. 3, 6).

Sodium deprivation in both intact and adrenalectomized rats resulted in almost complete loss of fat staining material from the pineal glands (Figs. 4, 5, 7). The weights of the pineal glands were less in sodium deficient intact rats ($P < .01$) and adrenalectomized rats ($P < .05$) when compared with their controls (table 1).

Observations on the distribution of fat in the pineal glands of intact control rats confirmed those reported by Quay (7) and will not be described here (Fig. 3).

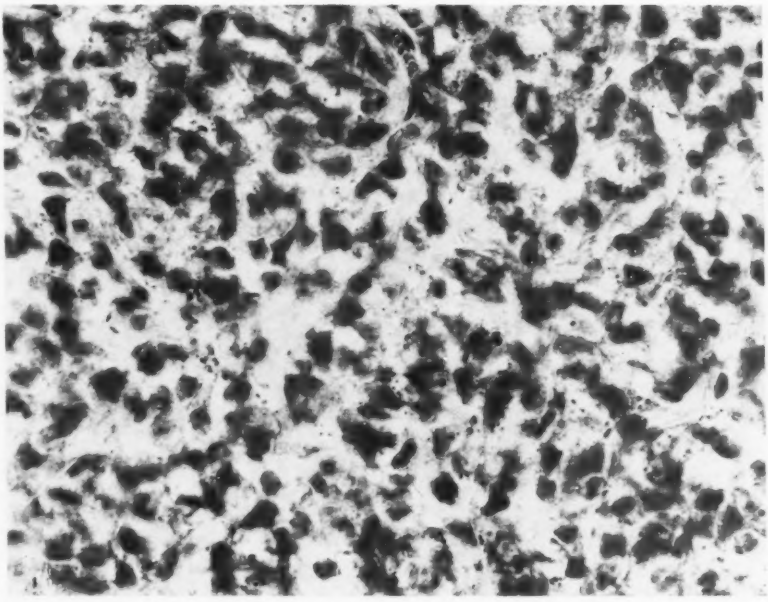

FIG. 4. Pineal gland taken from an intact rat on the sodium deficient diet for three weeks. The fat loss is only slightly greater than in Fig. 4. ×500.

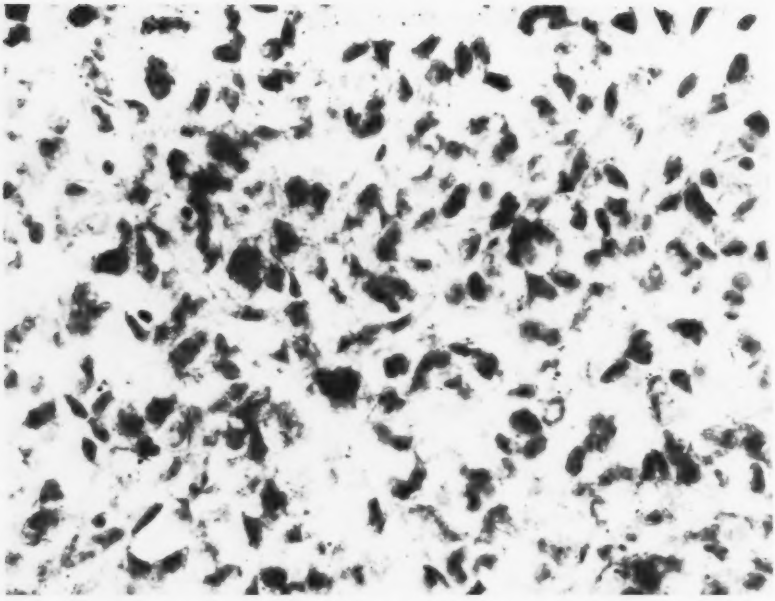

FIG. 5. Pineal gland taken from an intact rat on a sodium deficient diet for 1 week and demonstrates the severe loss of fat. ×500.

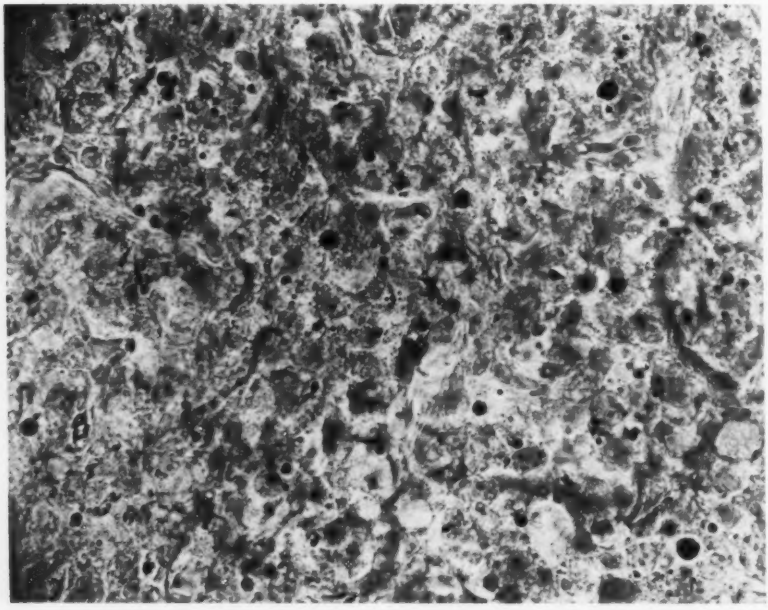

FIG. 6. Pineal gland taken from an adrenalectomized rat on the control diet and demonstrates a mild loss of fat. ×500.

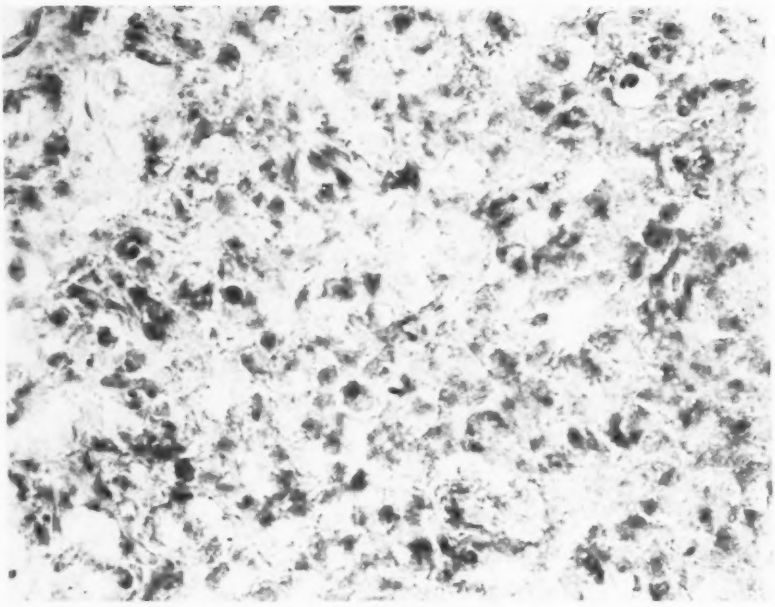

FIG. 7. Pineal gland taken from an adrenalectomized rat on the sodium deficient diet and demonstrates the most severe loss of fat. ×500

DISCUSSION

Morphologists have insisted for many years that the pineal body is indeed glandular in structure, although conclusions regarding the functional nature of the pineal gland have always been tenuous (8). The present results indicate that the pineal gland is affected by restricting the dietary sodium intake. Loss of stainable fat from the pineal gland in response to sodium restriction may indicate augmented activity of the pineal cells perhaps in releasing a hormone involved in salt regulation. Loss of fat from the adrenal cortex has been utilized as a measure of adrenal cortical activity following stress (9). An analagous phenomenon may occur in the pineal gland in response to salt restriction. In addition, adrenal ablation caused a mild loss of pineal fat, possibly indicating compensatory pineal activity due to lowering of body sodium levels. Loss of pineal fat may be only an associative phenomenon without functional significance and further experiments are planned to test the various possibilities suggested by these findings.

Farrell (3, 4, 5) has postulated that the pineal gland influences salt regulation by elaborating an "adrenoglomerulotropic" hormone which has its influence by acting through the zona glomerulosa of the adrenal gland. The amount of aldosterone was increased in adrenal vein blood by administering a pineal extract (a lipid-containing fraction). On the other hand, restriction of dietary sodium intake in pinealectomized dogs was effective

in producing the usual widening of the zona glomerulosa and augmented the amount of aldosterone in adrenal vein blood. Therefore, the pineal gland is not an essential step in stimulating the secretion of aldosterone but still may have an influence. The present report correlates well with the findings reviewed above. The pineal gland definitely responded to restriction of dietary sodium by loss of fat staining material but pinealectomy did not prevent the usual response of the zona glomerulosa of the adrenal gland under these conditions.

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GROWTH HORMONE CONTENT OF PITUITARIES FROM RATS WITH HYPOTHALAMIC LESIONS[1]

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ABSTRACT

Growth hormone (GH) content of pituitaries from rats with extensive ventral hypothalamic damage was determined by bioassay using the tibial epiphysial width stimulating test. Decrease in growth rate (measured by body weight and body length), decrease in pituitary size and decrease in pituitary somatotrophin concentration were produced by the lesion. It was estimated that the amount of GH per pituitary was reduced to approximately 15% of the normal. Secondary lesion-induced deficiences of thyroxine, testosterone or of vasopressin were not the cause of this effect because adequate replacement treatment was given. An earlier study on GH content of pituitaries from adrenalectomized rats was cited which makes it unlikely that lesion-induced ACTH deficiency was the cause of low GH found in these experiments. The demonstration of low concentrations of GH in the pituitaries of rats with lesions is the first direct evidence that some phase of somatotrophin secretion (decreased synthesis or increased release or both) is affected by hypothalamic damage. Specificity of the effect remains to be established because the lesions which were used to interfere with growth and to lower pituitary GH involve a substantial portion of the primary plexus of the hypophysial-portal vessels.

THE growth of rats with massive ventral hypothalamic lesions has been shown in previous experiments to be abnormally slow even when lesion-induced deficiencies of thyroxine, testosterone and vasopressin are corrected by replacement treatment (1). One of the possible explanations of this phenomenon is that the lesion inhibits pituitary growth hormone (GH) secretion, a suggestion at present unsupported by direct experimental evidence, but consonant with a number of other observations in the literature (cf. 1). It was the purpose of the present experiments to measure growth hormone content of pituitary glands from rats in whom hypothalamic damage had produced substantial reductions in growth rate.

METHODS

Holtzman rats of the Sprague-Dawley strain were used in this experiment. They were housed under constant lighting conditions in a temperature controlled room ($26 \pm 1°$ C), were given water *ad libitum* and Purina laboratory chow. Hypophysectomized[2] rats, used for assay were in addition given supplements of bread and oranges

Received October 12, 1960.

[1] Supported by U. S. Public Health Service Grant B834.

[2] Hormone Assay Laboratories, Chicago.

Two pairs of bilaterally symmetrical lesions were placed in males using a Krieg-Johnson stereotaxic instrument. The first pair was made 1.0 mm. behind bregma, 0.5 mm. to each side of the midline and 0.3 mm. up from the base; the second 2.0 mm. behind bregma, 0.8 mm. to each side of the midline and at base level. The electrode tip was bared 1.0 mm. and current of 3 MA was passed for 30 seconds in each electrode position. These coordinates were chosen so as to give massive destruction of the anterior half of the hypothalamus and median eminence and of the ventromedial nuclei without producing damage to the pituitary stalk (1). Lesions of this distribution had been found in previous experiments to produce growth impairment (1).

Sham operated control animals were prepared by inserting the electrode into the cortex to a depth of 2 mm. without the passage of current. From the time of operation, the food intake of each rat with a lesion was measured, and a like amount offered to a paired control; thus every rat with a lesion ate as much as or more than the amount taken by the control. Operated rats who consumed daily less than 18 grams of food after the fourth post-operative day were discarded from the series. Beginning on the day after operation both operated and control animals were given a daily intraperitoneal injection of a hormone supplement of l-thyroxine, 5 μg/100 gms. body weight and testosterone propionate 100 μg/100 gms. body weight, as previously described (1). When present, diabetes insipidus was treated by the once daily intramuscular injection of Pitressin tannate in oil in a sufficient quantity to bring water intake to within the normal range. The sham operated member of the pair was similarly injected with the same material. Measurements of body weight and length (nose-tail) were made at the time of operation and at the time of sacrifice. Two weeks after the lesion had been produced, the animals were anesthetized with ether and exsanguinated from the abdominal aorta into heparinized tubes. The brains and pituitaries were removed en bloc, and examined with the aid of a dissecting microscope in order to determine the position of the lesion and the condition of the pituitary stalk. The pituitary was then carefully removed, the anterior lobe dissected free, weighed to the nearest 0.1 mg. on a torsion balance and frozen at a temperature of $-10°$ C for later use in bioassay. The brains were fixed in formalin for subsequent gross examination. Pituitaries were selected for assay from animals in which at least one-half of the anterior median eminence had been destroyed and were not used if the stalk proper had been involved by the lesion.

Assay for growth hormone concentration was done by a modification of the method of Greenspan *et al.* (2). Hypophysial tissue from rats with lesions and from sham-operated, pair-fed controls was pooled by groups and homogenized in 0.9% saline. Appropriate dilutions were made to provide 3 dosage levels in the same volume of injected fluid (0.5 ml.). The diluted material was kept frozen until immediately before injection. Four daily injections were given to the hypophysectomized assay animals and on the 5th day these rats were killed and the right tibiae prepared for epiphysial width measurements. Eight to ten determinations were made on each bone. Instead of using the standard curve technique of bioassay, the potencies of pituitary extracts from lesion and control animals were calculated from a six point assay using the statistical design of Bliss (3).

RESULTS

1. *Growth in the Rats with Lesions*

Significant reduction in growth was produced by the lesions despite the fact that the animals with brain damage consumed as much as or more than the food taken by the controls (Table 1). Pituitary weight was also reduced.

TABLE 1. GROWTH AND PITUITARY WEIGHT IN RATS WITH HYPOTHALAMIC LESIONS (sacrificed 14 days after operation)

Group	No. of animals	Weight gain		Length gain		Pituitary weight	
		gm.	± S.E.	cm.	± S.E.	gm.	± S.E.
Lesion	19	44.6	4.9	1.5	.16	5.9	.3
Paired Control	21*	69.0	6.4	2.5	.22	8.5	.2
Statistical comparison, lesion vs. control (P values**)		$<.02$		$<.001$		$<.001$	

* Two extra paired controls were available because of the death shortly before the end of the experiment of the operated members of the pairs.
** Statistical evaluation made by methods described by Snedecor (16).

2. *Growth-stimulating Activity of Pituitary Extracts from Rats with Hypothalamic Lesions*

Pituitary extracts from control and lesion bearing animals were administered at 3 dosage levels (total dose over a 4-day period: 0.63, 1.25 and 2.5 mg. respectively) (Fig. 1). Validity of the assay was estimated statistically by the method of Bliss (3) from measurements in 45 assay animals (5–9 animals per point). The slopes of the log-dose curves were proved to be parallel and the errors in observations for the two curves were shown to be homogeneous. The potency ratio for the pituitary extract from rats with lesions as compared with extracts from normal animals was 0.20 and fiducial limits for the potency ratio at the 95% level of confidence were between 0.05 and 0.84. Mean epiphysial widths of assay rats receiving extract of control pituitaries was significantly greater than the mean value in assay animals receiving pituitary tissue from rats with lesions ($P<.001$). These data demonstrate that pituitary growth hormone content is depressed in rats with lesions. If one considers that the potency ratio is 0.2 and that the

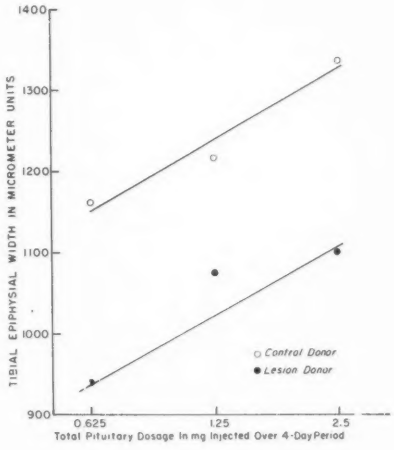

FIG. 1. Illustrated is the 6-point assay of growth hormone concentration in pituitaries from rats with hypothalamic lesions and from paired controls.

pituitaries from rats with lesions are significantly smaller than normal (0.70 times the weight of controls), it is apparent that growth hormone content in individual pituitaries of rats with lesions is about 15% of the normal.

DISCUSSION

Tibial epiphysial width stimulating activity of pituitaries from rats with hypothalamic lesions has been shown in this experiment to be significantly reduced when calculated either per milligram tissue weight or per unit pituitary. In view of previous studies on the epiphysial width test (2), there seems little reason to doubt that the hypothalamic lesions led to a decrease in pituitary growth hormone content. Comment should be made on the specificity of this assay as a test for GH in crude extracts of pituitary tissue as certain of the other trophic hormones affect responses to somatotropin. Thyrotrophin acts synergistically (2) but can be excluded from consideration as affecting the response because the donor animals, both normal and with lesions, were given thyroxine in a dosage which would reduce pituitary TSH to extremely low levels (4). Adrenotrophin which is antagonistic to GH in the tibia test (2), is present in abnormally small amounts in rats with hypothalmaic lesions (5). Therefore, any effect exerted by ACTH would be in the direction of reducing the effectiveness of the extract from the normal animals and could not be the cause of the greater poteney of the normal glands.

Associated lesion-induced pituitary deficiencies conceivably could account for the abnormal content of pituitary GH. Any effect exerted by lack of thyroxine, testosterone or vasopressin are readily excluded by the use in this experiment of appropriate hormonal replacements. Thyroid hormone is particularly important in this connection since there is good evidence that thyroxine deficiency causes a lowering of pituitary GH content (6). The one lesion-induced hormonal deficiency that cannot be easily excluded as a cause of pituitary GH depression is that of adrenocorticotrophin. The major experimental observation which makes this possibility unlikely is that adrenalectomized rats whose sodium deficiency has been treated by saline drinking water have pituitary GH concentration (by the tibial cartilage test) indistinguishable from that of pair-fed controls (7).

The finding of reduced pituitary GH in these animals is the first direct evidence that certain hypothalamic lesions can influence some phase of pituitary growth hormone secretion. Lowered concentration indicates either increased release or decreased synthesis or both; definitive proof that the lesions cause a reduction in GH secretion would require measurements of plasma GH concentration and of peripheral GH degradation rate. Preliminary studies of "sulfation factor" (8) in the plasma of these animals

done in collaboration with Dr. William Daughaday have been inconclusive.

In previous studies (1, 9) the various factors that might contribute to poor growth in rats with lesions were enumerated and discussed. To that list may now be added decreased pituitary growth hormone concentration. The hypothesis suggested by these data is that the hypothalamus can influence GH secretion in a manner analogous to the control exerted over other pituitary trophic hormones, ACTH, TSH and the gonadotrophins. This view still requires substantial additional supporting data. Even if it could be proved that GH secretion rate was reduced in these animals the problem of specificity of the effect of the lesions must still be considered. The question of interference with blood supply is a particularly formidable question, one that cannot be readily answered with respect to GH regulation in view of the fact that the lesions which are effective in reducing growth rate do, for the most part, damage at least the anterior half of the primary plexus (1, 9). Lesions of this distribution were used in the present experiments. Stress effects must also be considered. Acute trauma is known to cause an increase in pituitary GH content (6), but there is no evidence bearing on the problem of chronic stress with which the present data may be compared.

It is also known that direct hypothalamic pituitary connections are not essential for growth. Hertz has shown that substantial growth in rats is stimulated by pituitary grafts provided enough tissue has been used (10). Significant autonomy of pituitary growth hormone secretion has thus been demonstrated. The evidence presented and cited in this paper is suggestive of a hypothalamic regulatory influence on somatotrophin secretion. Proof of the specificity of the effect awaits further investigation.

If the effect of the hypothalamic lesions on growth hormone proves to be specific, the identity of the hypothalamic neurohumor which influences GH (presumably analogous to the corticotrophin releasing factor 11, comes next into question. The most direct evidence on this point was presented by del Vecchio, *et al.* (12) who stated that Pitressin injections increased the tibial epiphysial width in young rats. Hiroshige and Itoh (13) have made similar observations, and also state that liver RNA levels rise in Pitressin treated rats. These data suggest a role for vasopressin or a non-vasopressin constituent of Pitressin in the regulation of GH release. Moreover it has been shown that growth failure in rats with lesions is roughly proportional to the degree of polyuria produced (1, 9). On the other hand, the importance of vasopressin in GH release is questionable for several reasons. Replacement therapy with Pitressin tannate does not restore growth to normal in rats with lesions (1). In a recently completed study the protocol of del Vecchio, *et al.* was followed exactly (14); evidence of a STH releasing effect of Pitressin was not obtained. A clinical study of children treated for

craniopharyngioma indicates that surgically produced diabetes insipidus did not prevent normal growth in some cases (15).

Acknowledgments

Statistical analysis of the bioassay data presented in this paper was carried out by Mrs. Barbara Bartels Hixon whose help is gratefully acknowledged. Mr. Joseph G. Brown gave valuable technical assistance.

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THE EFFECT OF PROBENECID AND HYDROCHLOROTHIAZIDE ON THE CLEARANCES OF FREE AND CONJUGATED 17-HYDROXYCORTICOSTEROIDS, TOTAL 17-KETOSTEROIDS, DEHYDROEPIANDROSTERONE AND ANDROSTERONE

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ABSTRACT

The effect of administration of Probenecid and hydrochlorothiazide on renal clearances of free and conjugated 17-OHCS, total 17-KS, androsterone (AND) and dehydroepiandrosterone (DEA) has been determined in normal human subjects.

The data obtained suggest that the administration of Probenecid causes a sharp fall in the clearance of the 17-KS and mainly of AND, a less marked decrease in the clearance of the conjugated 17-OHCS, and no modification of the clearance of free 17-OHCS.

The administration of hydrochlorothiazide in contrast, resulted in a marked increase in the clearance of all steroids measured, particularly AND and conjugated 17-OHCS.

In the patients treated with long-acting ACTH and hydrochlorothiazide, the hormonal clearances, already high at the beginning, as a result of ACTH administration, tended to increase to even a greater extent as compared to the patients receiving hydrochlorothiazide alone, especially in the case of AND.

The authors have drawn the conclusion that it is possible to influence the plasma levels of steroids by pharmacological agents acting at the renal level.

IN PREVIOUS papers data were presented concerning the metabolic behaviour and the renal clearances of 17-OHCS, total 17-KS, androsterone (AND) and dehydroepiandrosterone (DEA). In addition, the effect of long-acting corticotrophin (ACTH) and of cortisone, administered to patients with or without adrenocortical diseases, on renal clearances of these steroids were studied (1, 2, 3, 4).

In the same papers we have shown that the plasma and urinary values of the steroids and their clearances represented not only the function of the adrenal gland, but also of the peripheral factors: renal, hepatic and tissue, capable of affecting the inactivation and the excretion of hormones. We have noted, in addition, the remarkable quantitative and qualitative variations in plasma and urinary levels of steroids and in their clearances that some hormones such as ACTH, cortisone and mainly aldosterone and diuretic drugs such as chlorothiazide exert on the kidney (3, 4, 5).

Received October 14, 1960.

The present work was undertaken in order to test the effects of pharmacological agents which influence renal tubular function (probenecid, which blocks tubular secretion and hydrochlorothiazide, is a strong diuretic) on the renal clearance of steroids and to compare these results with our previous observations.

MATERIALS AND METHODS

The investigations were carried out on 12 normal men ranging in age from 20 to 30 years. Standard clearance technics were used as described in previous papers (2, 3, 4). Renal clearances of hormones were studied by determination of plasma and urinary levels of total 17-KS, AND and DEA, of free and conjugated 17-OHCS, and by calculation of the clearances in relation to 6-hour periods. The free and the conjugated 17-OHCS were determined in plasma and in urine by our modification (6) of the methods of Nelson and Samuels (7) and Glenn and Nelson (8). The total 17-KS, AND and DEA were determined by our modification (9) of the method of Gardner (10). The urinary and plasma creatinine were measured by the method of Bosnes and Tousky (11) partially modified by Azerard (12).

The clearances tests were performed on three different groups of four persons:

In the first group the experiments were carried out for 6-hour periods (from 6 to 12 o'clock in the morning) of two successive days, before and after administration of probenecid. The probenecid was administered orally in a dose of 2 gm. (1 gm. six hours before and 1 gm. at the beginning of the second test of clearance).

The second group of patients was studied with the same methods as the previous one, before and after administration of hydrochlorothiazide, the drug being administered orally in a dose of 100 mg. (50 mg. six hours before and 50 mg. at the beginning of the second test of clearance).

The third group of four persons was given a long-acting ACTH preparation (in phosphate of poliphloretin), by intramuscular injection as described previously (10 I.U. every six hours) (2, 3, 4). In normal men high and constant plasma levels of 17-

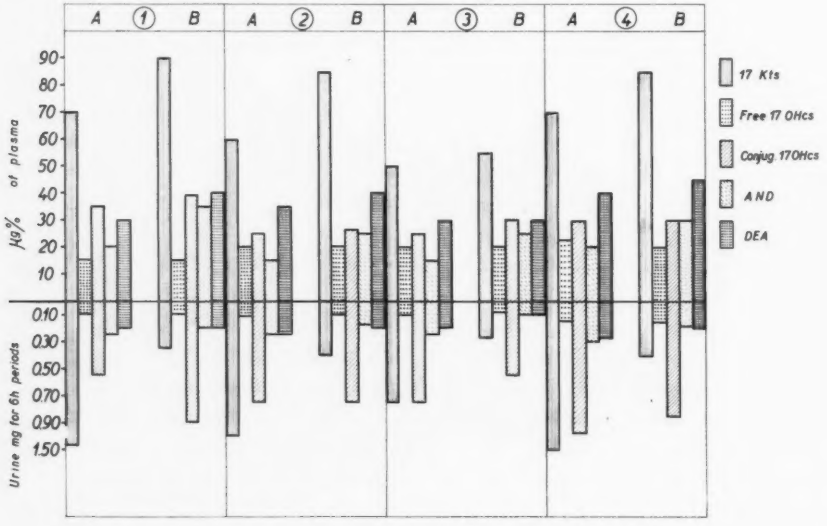

FIG. 1. Probenecid: A—before. B—after probenecid.

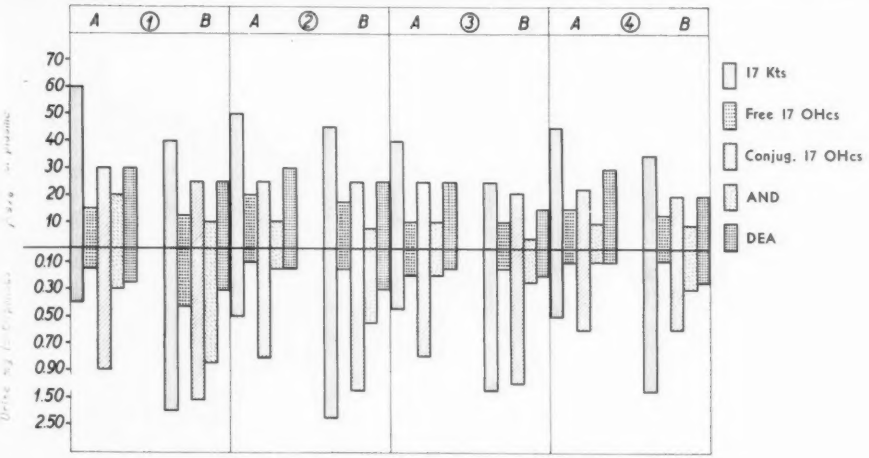

FIG. 2. Hydrochlorothiazide: A—before. B—after hydrochlorothiazide.

OHCS and 17-KS were obtained in this way for as long as nine hours after administration.

The renal clearances of steroids, in this investigation, were measured for two 6-hour periods, 12 and 24 hours after the beginning of the administration of ACTH. In the second period of six hours, hydrochlorothiazide (100 mg.) was added to ACTH. It was administered in a dose of 50 mg. six hours before, and 50 mg. at the beginning of the second test of clearance.

In all three groups, the blood samples were taken during the middle of the urine collection period.

RESULTS

The results of our researches are summarized in three figures and one table.

Figure 1 shows the plasma and urinary values of steroids in patients before (A) and after (B) administration of probenecid.

Figure 2 shows the plasma and urinary values of steroids in patients before (A) and after (B) administration of hydrochlorothiazide.

Figure 3 shows the plasma and urinary values of steriods in patients after administration of ACTH (A) and ACTH plus hydrochlorothiazide (B).

In Table 1, part (1°), the clearance values of fractions of steroids, studied before (A) and after administration of probenecid (B), are reported. The administration of probenecid causes a sharp fall in the clearance of the 17-KS and mainly of androsterone, a less marked decrease in the clearance of the conjugated 17-OHCS, and no modification of the clearance of free 17OH-CS.

In part two (2°) of the table, the clearance values of steroids of the second group of subjects examined, before (A) and after administration of hydrochlorothiazide (B), are reported. In these subjects, in contrast to

TABLE 1

		1° A = Before B = After probenecid				2° A = Before B = After hydrochlorothiazide				3° A = After ACTH B = After ACTH + hydrochlorothiazide			
		Free 17-OHCS clearance % creatinine clearance	Conjugated 17-OHCS Clearance % creatinine clearance	17-KS clearance % creatinine clearance	AXD clearance % creatinine clearance	DEA clearance % creatinine clearance	Free 17-OHCS clearance % creatinine clearance	Conjugated 17-OHCS clearance % creatinine clearance	17-KS clearance % creatinine clearance	AXD clearance % creatinine clearance	DEA clearance % creatinine clearance	Free 17-OHCS clearance % creatinine clearance	Conjugated 17-OHCS clearance % creatinine clearance	17-KS clearance % creatinine clearance	AXD clearance % creatinine clearance	DEA clearance % creatinine clearance
1	A	1.70	7.20	5.30	3.90	1.90	0.30	7.90	1.90	4.10	1.00	2.10	17.10	4.95	10.50	2.00
	B	1.40	8.00	1.00	1.50	1.20	0.90	19.00	14.00	23.00	3.20	2.30	34.10	30.00	40.00	5.00
2	A	1.90	8.00	3.80	4.00	1.50	0.45	8.00	2.80	3.85	1.10	1.25	14.00	4.90	15.90	1.98
	B	1.80	6.50	0.20	1.70	1.00	2.00	13.50	10.50	23.10	2.00	1.70	22.10	20.00	78.50	5.50
3	A	6.90	6.80	3.50	4.50	1.70	0.55	9.10	3.00	4.10	1.20	2.00	18.50	5.10	12.00	2.50
	B	1.20	5.50	1.00	1.00	0.95	1.90	16.50	15.50	24.50	2.90	2.40	36.00	19.80	50.00	4.80
4	A	1.80	7.70	6.00	4.10	1.80	0.35	7.50	3.00	3.20	0.98	1.90	16.00	6.00	11.11	3.00
	B	2.00	6.10	1.20	1.10	1.00	1.00	9.10	9.10	21.00	2.80	2.15	27.00	22.00	38.50	6.10

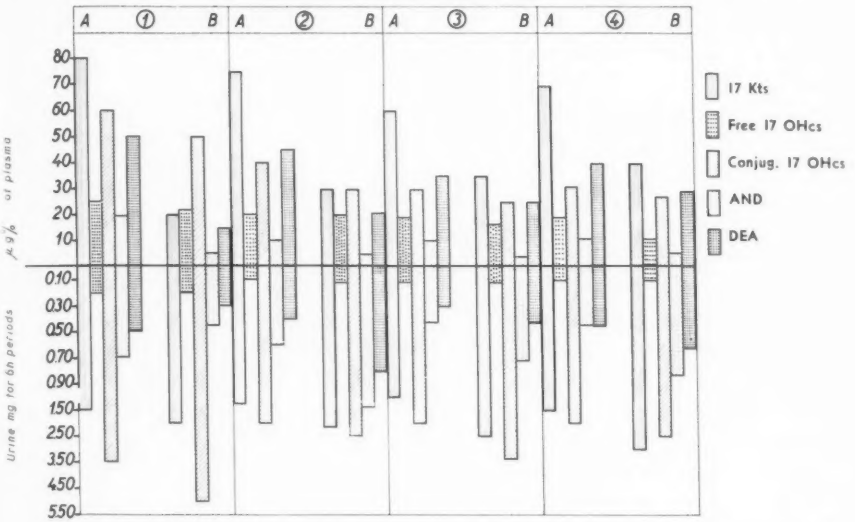

FIG. 3. ACTH+hydrochlorothiazide. A = ACTH. B = ACTH+hydrochlorothiazide.

what was observed in the first experiment, diuretic administration, resulted in a marked increase in the clearance of all steroids measured, particularly AND and conjugated 17-OHCS.

In part three (3°) of the table the patients treated with long-acting ACTH and hydrochlorothiazide are listed. In these subjects hormonal clearances, already high at the beginning as a result of ACTH administration, tended to increase to even a greater extent as compared to the patients receiving hydrochlorothiazide alone, especially in the case of AND.

DISCUSSION

Probenecid appears to cause an inhibition of certain functions of the renal tubules. This inhibition has been used for a long time in the treatment of gout, because of its effect on inhibiting the tubular reabsorption of uric acid. It has also recently been used in the treatment of hypoparathyroidism because it blocks the tubular reabsorption of phosphorus. Probenecid, moreover, causes a decrease in the urinary excretion of some endogenous or exogenous substances which, like penicillin and paraaminobenzoic acid, are removed by kidney via tubular secretion (13). Since some steroids, mainly AND, are excreted in the urine by means of tubular secretion, their excretion is inhibited by probenecid (14, 15, 16, 17).

Our experiments with probenecid, carried out with different methods from those used by the above authors (14, 15, 16, 17) confirm such behaviour with regard to the 17-KS of normal endogenous production.

It is interesting to point out that hydrochlorothiazide causes effects very

different from those of probenecid, as far as the clearance of the steroidal hormones is concerned; hydrochlorothiazide, not only remarkably increases the clearance of the endogenous 17-KS, mainly of AND, but also the clearance of the conjugated 17-OHCS to a greater extent than was found for chlorothiazide (3, 4, 5). It is well known that hydrochlorothiazide, probably by decreasing tubular reabsorption of NaCl, has a remarkable diuretic effect. We think that hydrochlorothiazide may increase steroid excretion by a similar mechanism. It is possible that the increase in urinary excretion of the hormones could be connected with a greater elimination of water.

Some years ago, McHenry *et al.* (18) and Pincus (19) pointed out that there might be a relation between the urinary volume and the excretion of the 17-KS in normal men. Recently Brown and Asher (20) found an increase in the urinary excretion of steroids and a decrease in plasma levels in normal subjects to whom water had been administered. The authors attributed the above mentioned result to a direct effect of water on renal function rather than indirectly through stimulation of adrenocortical secretion.

In a recent paper Hatfield and Shuster (21) found no increase in the excretion of conjugated 17-OHCS, whereas they observed a remarkable increase in urinary values of 17-KS and of free 17-OHCS in subjects who had undergone water administration. The above-mentioned authors suggest, on the basis of their results, that the increase in the excretion of these steroids after water administration may be due to an action on renal tubules. These experiments show that the homeostasis of steroids can be influenced by agents acting at the renal level.

In the patients we studied, a close relation between urinary volume and excretion of steroids was not found. Administration of hydrochlorothiazide did not cause the same changes in plasma and urinary levels of the steroids examined; in fact hydrochlorothiazide, exerting at renal level, caused a more remarkable increase in the urinary excretion of 17-KS than in the excretion of 17-OHCS, and caused a more remarkable increase in the AND urinary excretion than in the DEA excretion. Also, the hydrochlorothiazide caused an increase in urinary excretion of conjugated 17-OHCS and a less or no increase at all of free 17-OHCS excretion.

The levels of steroids in the blood of the normal individuals we have studied undergo, after administration of hydrochlorothiazide, a remarkable decrease without reaching, however, levels lower than those of normal values. In subjects to whom long-acting ACTH was given, the administration of hydrochorothiazide caused an increase in the clearances of steroids.

In our previous papers (2, 3, 4) we emphasized that the increase of the clearance of steroids caused by ACTH and cortisone, was due mainly to an increase in glomerular filtration of the hormones. This makes it possible to influence the urinary excretion of steroids even in subjects with adreno-

cortical hyperfunction. The steroidal values, in the blood of subjects to whom ACTH had been given, undergo a decrease after administration of hydrochlorothiazide which is proportional to that found in subjects not treated with ACTH. However the values of steroids in plasma in the above mentioned subjects were still higher than normal.

These data suggest, in agreement with Hatfield and Shuster (21), that it is possible to influence the plasma levels of steroids acting at renal level, but changes in plasma levels of steroids will occur only if the adrenal cortex exerts a permissive action.

In cases of adrenal hypofunction, the administration of hydrochlorothiazide might cause a slight hormonal compensation with low plasma value of steroids. On the other hand, in states of adrenal hyperfunction plasma values of steroids unmodified or higher than those at the outset might be found after the administration of this diuretic drug.

Acknowledgment

We would like to thank CIBA who supplied us with the drug "hydrochlorothiazide" (Esidrex).

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THE EFFECT OF DIETHYLSTILBESTROL ON THE IMMATURE RAT OVARY[1]

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ABSTRACT

Hypophysectomized and intact immature female rats were injected with diethylstilbestrol in daily doses of 1 mg. or 1 μg for periods of 2–20 days.

The large doses increased ovarian weights to an average of 37 mg. on the 8th day in both the intact and hypophysectomized groups, after which a gradual decline occurred for the duration of the experiment. In hypophysectomized animals, ovarian weights were significantly greater than those of uninjected controls for the entire experimental interval. This was true in intact animals until 11 days, after which control ovaries were heavier owing to luteinization.

The small dose of stilbestrol did not significantly increase ovarian weight in hypophysectomized rats, although some follicular growth was noted microscopically. In intact rats, the small dose increased ovarian weights at 8 days, apparently through intermediary release of pituitary gonadotrophin.

This study confirms previous investigations in showing that large doses of stilbestrol cause growth and maintenance of medium-sized follicles in rat ovaries. The demonstration of similarities in weight increases and histological findings in intact and hypophysectomized rats provides further evidence for a direct action of stilbestrol on the ovary.

MOORE and Price (1) injected small doses of sex hormones into immature rats and concluded that these substances exerted no direct influence on the gonads. Pencharz (2) and Williams (3) implanted stilbestrol pellets subcutaneously in hypophysectomized immature female rats and noted increased numbers of medium-sized follicles and maintenance of ovarian weight after eight days. Similar results were obtained by Payne and Hellbaum (4) after injecting large doses of stilbestrol into hypophysectomized rats for four days. These results indicate that stilbestrol exerts a direct effect on the rat ovary. The present study was undertaken to investigate this influence with special regard to duration of treatment and comparison of large and small doses.

MATERIALS AND METHODS

Twenty-three-day-old female Sprague-Dawley rats, hypophysectomized at Hormone Assay Laboratories, Inc., Chicago, were received in this laboratory on the second postoperative day. Each animal was injected with 2.5 mg. cortisone acetate upon arrival and injections of diethylstilbestrol were begun on the following day.

Received October 24, 1960.

[1] This study was supported by U.S.P.H.S. Grant A-1686.

Intact female rats were received at 23–24 days of age and injections were begun on the following day.

Each test or control group was composed of 5 animals. Test animals were injected subcutaneously once daily with either 1 mg. or 1 μg of diethylstilbestrol dissolved in 0.1 cc. of peanut oil. The injections were continued for various periods ranging from 2 to 20 days. Uninjected control animals were caged separately. All animals had access to Purina chow and tap water *ad libitum*.

Twenty-four hours after the last injection, each test group of rats was asphyxiated with natural gas. Control groups were also killed at corresponding times. The ovaries were dissected free, weighed to the nearest 0.5 mg. on a Roller-Smith torsion balance, and fixed in Bouin's solution. After paraffin embedding, the ovaries were sectioned at 5 μ and stained with hematoxylin and eosin. Initial and terminal body weights were recorded and the condition of the uterus was noted at autopsy.

RESULTS

Figure 1 shows the averages for ovarian weights of 70 hypophysectomized rats after various intervals of either observation or treatment with stilbestrol. It is evident that ovarian weight is more than tripled after 8 days of treatment with 1 mg. doses. Even though a gradual decline followed, at 20 days of treatment the ovarian weight was still significantly above that of the initial and terminal controls. The ovaries of uninjected control animals declined in weight after hypophysectomy and those animals injected daily with 1 μg of stilbestrol had ovaries weighing only slightly more than the controls.

Histologically, the ovaries contained many medium sized follicles after treatment with a total of 8 mg. (Fig. 3). Some follicles showed atretic changes, but many were healthy and growing. After 8 μg of stilbestrol, the ovaries contained more growing follicles than did control ovaries although the degree of stimulation was minimal (Figs. 4 and 5).

Figure 2 shows the averages for ovarian weights of 105 intact animals used in this study. Ovarian weights were double those of controls after

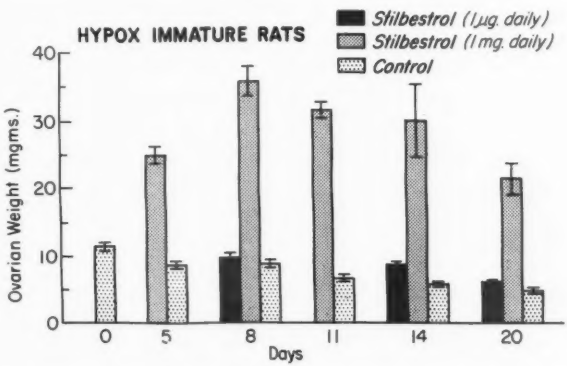

FIG. 1. Average ovarian weights of hypophysectomized rats. Brackets indicate ± standard error of the mean.

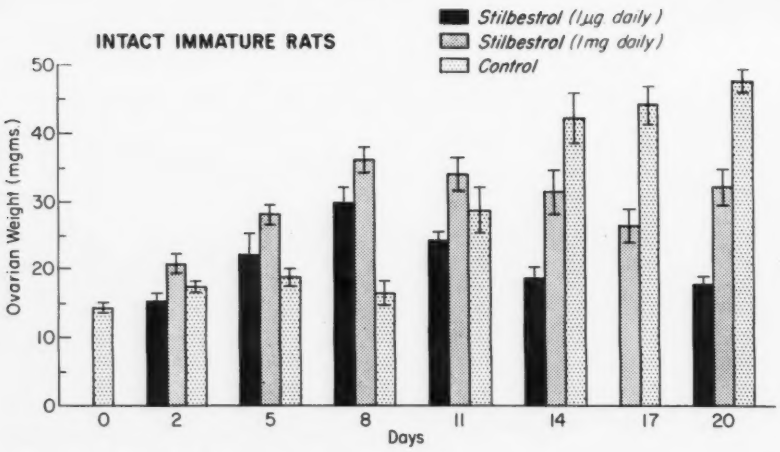

FIG. 2. Average ovarian weights of intact rats. Brackets indicate ± standard error of the mean.

8 days of treatment with 1 mg. doses. Longer intervals were associated with regression in ovarian weight. A similar pattern is noted for intact animals receiving 1 μg daily. After 11 days, ovaries of control animals uniformly contained corpora lutea and large follicles and were heavier than ovaries of treated animals.

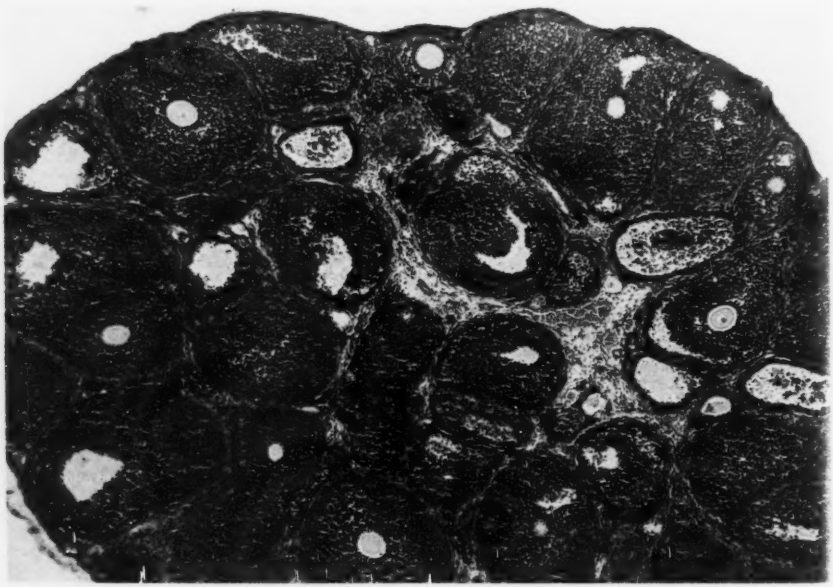

FIG. 3. Ovary from hypophysectomized immature rat after 8 days of treatment with 1 mg. stilbestrol daily.

FIG. 4. Ovary from hypophysectomized immature rat after 8 days of treatment with 1 μg stilbestrol daily. There are more growing follicles than in the uninjected control (Fig. 5).

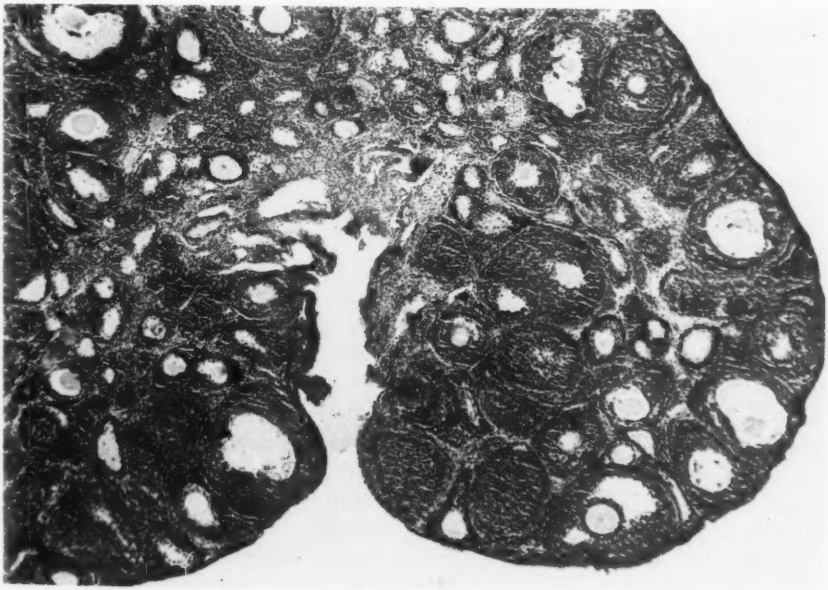

FIG. 5. Ovary of uninjected hypophysectomized control rat at 8 days.

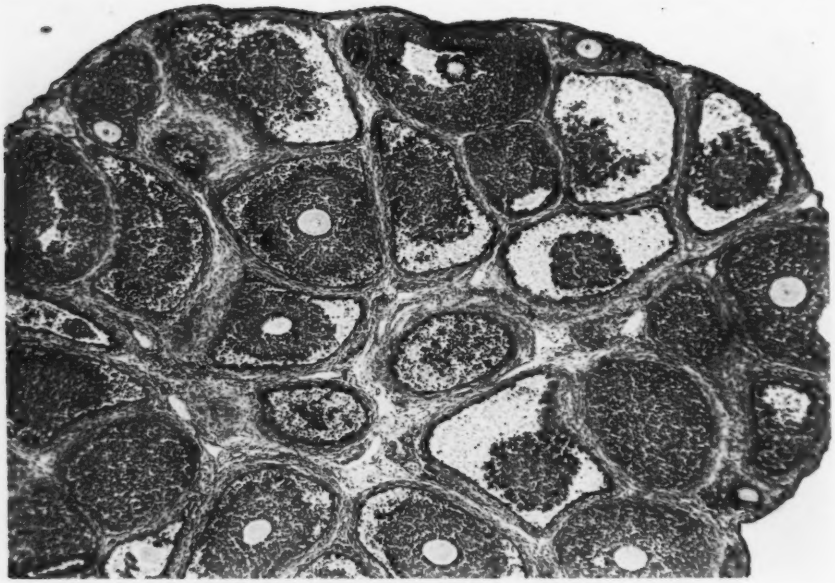

FIG. 6. Ovary of intact immature rat after 8 days treatment with 1 mg. stilbestrol daily. The peculiar peripheral dissolution of the granulosa was described by Simpson *et al.* (5).

Histologically, the ovaries of intact rats given a total of 8 mg. stilbestrol were very similar to those of the hypophysectomized rats receiving the same dose (Figs. 3 and 6). Control ovaries at 8 days were immature in appearance and contained few growing follicles (Fig. 7).

Body weights of animals treated daily with 1 mg. stilbestrol were lower than those of animals receiving 1 μg daily or untreated controls. If the ratio of ovarian weight to body weight had been computed (4), the apparent difference between the effect of the large and small dose on ovarian weight increase would have been even greater.

DISCUSSION

Ovarian reponse in hypophysectomized rats

The results noted after treatment with daily 1 mg. doses of stilbestrol confirm earlier observations. Payne and Hellbaum (4) and Simpson *et al.* (5) produced enlarged ovaries containing many medium-sized follicles after 4 and 8 days respectively. It was found that 1 mg. daily of stilbestrol was about the maximal dose tolerated by immature rats (4). Gaarenstroom and de Jongh (6) injected 1 mg. estradiol benzoate daily for 5 days after unilateral castration and noted a slightly increased weight of the remaining ovary.

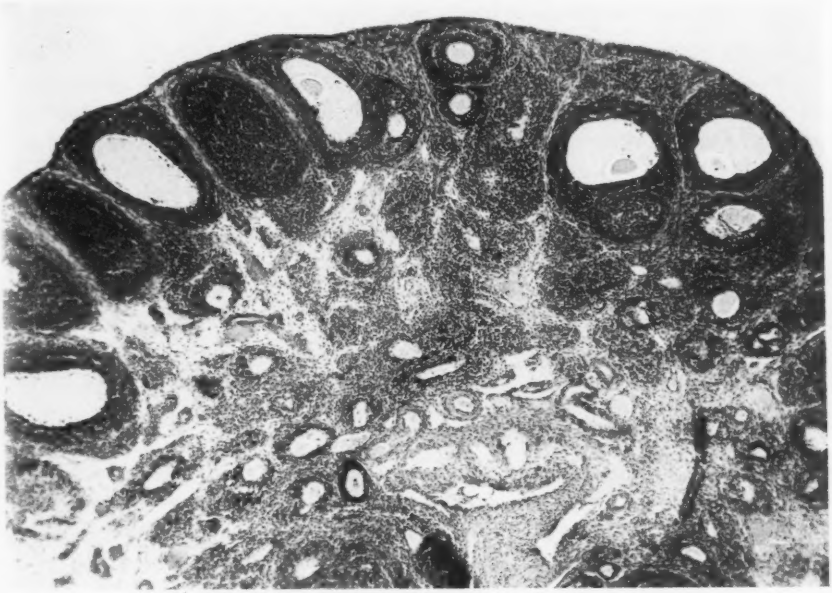

FIG. 7. Ovary of intact uninjected control rat at 8 days.

Our particular interest in the present study was to elucidate the sequential pattern of follicular growth and ovarian weight change during continuous estrogen administration. The period of increasing ovarian weight appears to be limited to about the first 8 days of treatment, by which time maximum weights were obtained in this particular series. A gradual decline had begun by 11 days, and although a relatively wide range was observed at 14 days as indicated by the standard error, this decline continued through 20 days. However, it is noteworthy that the large dose treatment maintained ovarian weights well above control levels for the entire experimental interval.

The small dose did not prevent post-hypophysectomy ovarian weight regression. This was in marked contrast to the weight stimulating effect of the large dose. Apparently, small doses do not reach the ovary in sufficient concentration to cause significant weight increases, but a slight rise in numbers of solid, medium-sized follicles after 8 days of 1 μg stilbestrol daily indicates some direct effect. Other investigators have used small doses of estrogens in attempts to influence the ovary directly. Greep and Jones (7) and Paesi (8) found no appreciable weight change after 3 to 7 days treatment with daily doses of estradiol benzoate smaller than 1.4 μg. Increased average follicular size was noted by Croes-Buth *et al.* (9) after injection of doses as small as 0.5 μg estradiol benzoate, but ovarian weight increases were not evident.

Ovarian response in intact animals

No previous study has considered the sequential changes in the ovaries of intact immature rats during continuous estrogen treatment. The controls selected for comparison in this type of study are important since significant ovarian changes occur between the third and fifth weeks of life. Moore and Price made observations after a 3 week interval by which time untreated control ovaries contained corpora lutea. Their results were therefore similar to those for the small dose in the 14–20 day interval in the present study (Fig. 2). From this they generalized that estrogen inhibited the ovary by decreasing gonadotrophin release from the pituitary and concluded that estrogen had no direct effect on the ovary.

Our observations show that the above interpretation is invalid for earlier intervals if one considers the findings after 5–8 days of treatment with the small dose (comparable to that used by Moore and Price). At 8 days, ovarian weights were increased significantly over those of untreated controls (Fig. 2). This contrasts with the results noted after the same dose and interval in the hypophysectomized series where no appreciable weight changes occurred. We would not argue that the ovarian weight stimulation in the intact series was due to a direct effect of the small estrogen dose. Most of this effect is probably due to an early release of gonadotrophin by the pituitary. Fevold and Fiske (10) and Bradbury (11) have shown that small doses of estrogen increase the output of gonadotrophin in the 5–8 day interval. It seems likely that the gonadotrophin and estrogen exert a combined influence resulting in increased ovarian weight. A comparison of histological findings in the ovaries of intact and hypophysectomized rats treated with the small dose supports this interpretation. Large, vesicular follicles were predominant in the ovaries of the intact rat, whereas the ovaries of the hypophysectomized rat contained mostly solid, smaller follicles.

The ovarian weight increases produced by the large estrogen dose in intact rats are very similar to those observed in the hypophysectomized group, both in sequence and weight levels attained (compare Figs. 1 and 2). Some of the effect of large doses in the intact animals may conceivably be attributed to intermediary action by the pituitary. However, it seems more likely that, during large dose treatment, sufficient quantities of estrogen reach the ovary to produce direct follicular stimulation, as evidenced by the similar morphological response obtained in both intact and hypophysectomized rats (Figs. 3 and 6).

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EFFECT OF A STEROID SPIROLACTONE ON THE CARDIOVASCULAR CHANGES PRODUCED BY RENAL ISCHEMIA[1]

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ABSTRACT

SC-9420 (or Aldactone), an antimineralocorticoid spirolactone significantly protects against the mortality and some of the cardiovascular-renal changes normally produced by the presence of an ischemic (or "endocrine") kidney in rats.

Since Aldactone acts similarly on the manifestations of desoxycorticosterone overdosage, this, together with previous findings, indicates a close relationship between the toxic effects of mineralocorticoids and those of renal secretory products.

Steroid spirolactones may prove to be useful against cardiovascular damage in clinical cases of renal hypertension.

THE production of permanent unilateral renal ischemia in rats induces a characteristic pathological pattern in the cardiovascular system, indistinguishable from the changes seen after overdosage with desoxycorticosterone (DOC) (1). The similarity between the manifestations of renal ischemia and those of mineralocorticoid (MC) toxicity was further demonstrated by their identical response to pituitary and adrenal influences (2), and to the effect of certain Na-salts that modify the pathological picture in the heart (3). It has also been shown that both agents are able to aggravate nephrocalcinosis produced by excessive phosphate administration (4). Apparently the effect of the ischemic kidney is not mediated through the stimulation of adrenocorticoid secretion since adrenalectomy does not abolish its action (2).

On the basis of the obvious parallelism between the action of renal ischemia and MC overdosage it was of interest to see whether one of the newly discovered mineralocorticoid antagonists is able to influence the morphological changes elicited by unilateral renal ischemia.

MATERIALS AND METHODS

Fifty-three female Sprague-Dawley rats from the Holtzman farm, with an average initial body weight of 100 gm. (range: 92 gm.–109 gm.) were divided into four groups

Received October 28, 1960.

[1] This work was supported by the National Heart Institute, U. S. Public Health Service (Grant no. H-3688 (C2)).

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TABLE 1. ACTION OF ALDACTONE ON THE "ENDOCRINE-KIDNEY SYNDROME"

Group*	Treatment	Cardiac						Pancreatic periarteritis		Nephrosclerosis		Weight (mg.)†		Mortality %
		Periarteritis		Hyalinization		Necrosis								
		Incidence %	Severity† 0–3	Incidence %	Severity† 0–3	Incidence %	Severity† 0–3	Incidence %	Severity† 0–3	Incidence %	Severity† 0–3	Thymus	Adrenal	
1 (10)	None	0	0	0	0	0	0	0	0	0	0	276 ± 34	28.0 ± 2.0	0
2 (10)	Aldactone	0	0	0	0	0	0	0	0	0	0	242 ± 38	35.6 ± 2.8	0
3 (18)	"Endocrine kidney"	94	2.4 ± 0.2	100	2.2 ± 0.2	48	0.6 ± 0.2	94	2.0 ± 0.2	100	1.8 ± 0.2	127 ± 42	39.3 ± 2.4	48
4 (15)	Aldactone + "Endocrine kidney"	80	1.8 ± 0.2	33	0.5 ± 0.2	43	0.6 ± 0.2	43	0.9 ± 0.3	39	0.5 ± 0.2	234 ± 50	37.0 ± 2.1	0

* Number of animals indicated between parentheses.
† Mean ± standard error.

as indicated in Table 1. The animals were kept on "Purina Fox Chow" and tap water ad libitum. 3-(3 keto-7-thio-acetoxy-17β-hydroxy-4-androsten-17α-yl) propanoic acid lactone (SC-9420) or "Aldactone"[8] was given at the daily dose of 7 mg. in the form of a microcrystal suspension, in 0.2 cc. of water, subcutaneously, starting on the first day of the experiment. On the third day unilateral ischemia of the left kidney was produced according to the "endocrine-kidney technique" of Selye (5) by partially ligating the aorta between the two renal arteries, using the stylet of a 22 gauge hypodermic needle. On the eighteenth day the surviving rats were killed with chloroform, and the hearts, kidneys and pancreases were fixed with Susa solution and embedded in paraffin for subsequent staining with the periodic acid-Schiff reagent. The histological changes were graded on an arbitrary scale from 0 to 3, this being the most feasible way of measuring morphological results that cannot be expressed in more quantitative terms. The adrenal and thymus glands were weighed on a torsion balance. The microscopic findings, together with their standard errors, the percentual incidence of lesions as well as the mortality rate are shown in Table 1.

RESULTS AND DISCUSSION

Neither the intact control (group 1) nor the intact, Aldactone-treated rats (group 2) showed any pathological changes. In group 3, almost 50% of the "endocrine-kidney" bearing animals succumbed to severe cardiovascular and renal damage. This mortality rate was completely prevented by the steroid spirolactone (group 4). Most of the morphological lesions were also decreased by Aldactone, with the exception of cardiac periarteritis and occasional necrosis. The other manifestations of the "endocrine-kidney syndrome," namely myocardial hyalinization, nephrosclerosis and pancreatic periarteritis were significantly inhibited by the antimineralocorticoid. These observations might be of great significance from both a pathogenic and a clinical point of view. Our previous findings regarding the similarities between MC toxicity and the "endocrine-kidney syndrome" are now further supported by the fact that a substance known as a MC antagonist can also counteract a syndrome that has similar manifestations but is of a virtually different origin from that of MC overdosage.

Preliminary experiments performed on adrenalectomized rats indicate that SC-9420 does not exert its beneficial effect by merely suppressing the action of endogenous MCs; however a larger number of animals are still needed to affirm this within the limits of statistical significance.

Our findings are in accordance with those of other authors on the toxicity of DOC. Sturtevant has found that certain spirolactones markedly antagonize the effect of DOC on electrolyte changes while the vascular-renal damage is only slightly influenced (6, 7). He has shown that although SC-5233 prevents hypertension, some morphological lesions are still present. Selye (8) reported on the protective effect of Aldactone against the myocardial necrosis produced by 9α-fluorocortisol plus Na_2HPO_4. He also observed that even after treatment with Aldactone, small necrotic foci are still detectable in 50% of the rats, while the mortality is completely prevented. Ducommun *et al.* (9) have found that while Aldactone signifi-

antly decreases the mortality rate, it has no effect on the hypertension and nly partially prevents the pathological changes produced by DOC overlosage.

The prevention by Aldactone of thymic involution normally seen after he "endocrine-kidney" operation indicates that this compound tends to liminate the nonspecific stress effect of the ischemic kidney. The developnent of adrenal hypertrophy under these circumstances does not necessarly contradict this, since it might be due to the specific effect of the antimineralocorticoid which, by the inactivation of endogenous MCs, timulates the hypersecretion of these hormones, thus leading to adrenal ypertrophy, as seen in group 2 in which intact animals treated with Aldactone also have enlarged adrenals.

When comparing our present findings with those reported on the effect of steroid spirolactones upon the manifestations of DOC overdosage, one may conclude that there seems to be a close relationship between the action of mineralocorticoids and that of renal secretory products. Steroid spirolactones may prove to be useful against cardiovascular damage of patients with renal hypertension.

Acknowledgment

Aldactone was generously supplied by Dr. V. A. Drill, G. D. Searle and Co.

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INFLUENCE OF STRESS ON PLASMA AND ADRENAL CORTICOSTERONE LEVELS IN RATS WITH INTACT AND REGENERATING ADRENALS[1]

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ABSTRACT

A study was made of the ability of the regenerating adrenal to secrete corticosterone in response to stress as part of our investigation of the possible role of regenerating adrenocortical tissue in the genesis of adrenal-regeneration hypertension. Response to stress was measured in rats bearing intact adrenals, two regenerating adrenals, and one regenerating adrenal. Groups of 20 such animals were given water or 1% saline to drink, or uninephrectomized and given 1% saline. Twenty-one days after enucleation, the rats were decapitated under "resting" and "stressed" conditions. Corticosterone in plasma and adrenal pools was measured by an isotopic dilution technique using corticosterone-4-C^{14} and a fluorometric procedure. Plasma level of all groups of unstressed rats were essentially the same, ranging from 3 to 7 μg corticosterone/100 ml. In rats with intact adrenals, plasma corticosterone levels were directly related to the amount of steroid in the glands under resting and stressed conditions. Regenerating adrenals contained from 2 to 4 times more corticosterone under resting conditions than could be expected from plasma levels. Rats with two regenerating adrenals responded to stress with higher peripheral plasma levels of corticosterone than did animals with one regenerating gland, but these values were always less than those of the controls. Neither salt load, nor salt load and uninephrectomy changed the functional response of regenerating adrenal tissue to stress.

IN AN attempt to clarify the role of adrenal cortical tissue in the production of the hypertensive syndrome in rats bearing regenerating adrenals, a study of the ability of regenerating glands to secrete corticosterone in response to stress was undertaken. Holzbauer and Vogt (1) and Weisz and coworkers (2) have reported that regenerating adrenals secrete less than normal amounts of corticosterone for as long as 12 weeks after enucleation. Masson *et al.* (3) found that regenerating adrenals removed from uninephrectomized, salt-treated rats 3 and 4 weeks after enucleation produced less corticosterone than did normal glands when incubated with ACTH. Adrenal venous plasma from these animals also was found to contain smaller quantities of cortical hormones than did adrenal vein plasma

Received November 4, 1960.

[1] Supported by Research Grants from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service (H-3193) and the Edward G. Schlieder Educational Foundation, New Orleans, Louisiana.

rom control rats. On the other hand, Giroud *et al.* (4) reported normal orticosterone secretion by the regenerating adrenal after 30 days. Reconliation of these divergent findings may lie in the suggestion of Brogi and ellegrino (5) that in response to stress, regenerating adrenals can supply ormal amounts of corticosterone for short periods, up to 30 minutes, but e incapable of a high rate of secretion over longer intervals. It was felt that etermination of the levels of corticosterone in peripheral plasma and drenal tissue of animals bearing regenerating glands under both resting nd stressful conditions might help to resolve this issue of the functional apacity of these glands. Since regenerating adrenal glands have been mplicated in the genesis of a form of experimental hypertensive disease (6) it was considered worthwhile to similarly study the function of regenerating adrenals in animals given a 1% sodium chloride drinking solution and in others which were uninephrectomized and given 1% saline, the latter conditions being those necessary for the development of hypertension.

METHODS

Groups of 20 female rats of the Holtzman strain weighing 50 to 60 gm. were (A) left intact, (B) both adrenals enucleated, or (C) adrenalectomized on the right side and the left adrenal enucleated. In the first experiment animals of groups A, B, and C were fed Purina Chow and water ad libitum. In the second experiment 3 such groups were given 1% NaCl to drink, while in the third experiment all animals in the 3 groups were uninephrectomized and given 1% saline. The rats were killed after 21 days, because active regenerated cortical tissue is present at this time (7) and elevation of blood pressure generally begins between 3 and 4 weeks after enucleation (6). Non-stressed or "resting" conditions were attained by keeping the rats in individual cages in the dark overnight, after which they were carefully removed one at a time and rapidly decapitated in another room. Stress was induced by bilateral fracture of the tibia and fibula under ether anesthesia and the rats were killed 1 hour later. Peripheral blood was collected from the severed neck in heparinized beakers, centrifuged in the cold and the plasma removed and frozen. The adrenals were quickly dissected out, weighed and quick frozen.

Isotopic dilution with corticosterone-4-C^{14} [2] was selected at the best method for determining the minute amounts of the natural hormone present in plasma and adrenal tissue. Accordingly, from 2 to 10 μg of corticosterone-4-C^{14} of known specific activity was added to the pools of plasma or adrenals from each group. The plasma was diluted to 3 times its volume with water and extracted with 15 volumes of $CHCl_3$. This extract was washed with 0.05 volumes of 0.1 N NaOH and twice with 0.05 volumes of water. The $CHCl_3$ was removed in vacuo at 30° C using a rotary evaporator and the residue taken up in 70% methanol. The adrenals from each group were homogenized in the cold and extracted with hot alcohol-ether as previously described (8). The solvents were distilled off *in vacuo* and the residue dissolved in 70% methanol. These methanolic solutions were extracted 5 times with 2 volumes of hexane, in a procedure similar to that of Ulrich's (9). The hexane extracts were combined and washed with 0.05 volumes of 70% methanol. This wash solution was combined with the previous methanolic extract and the organic solvent removed *in vacuo*. The aqueous remainder was extracted 4 times with equal volumes of redistilled ethyl acetate and this extract reduced in

[2] Kindly supplied by the Steroid Allocation Committee of Endocrinology Study Section, U. S. Public Health Service.

TABLE 1. CORTICOSTERONE CONTENT OF PERIPHERAL PLASMA AND ADRENAL TISSUE IN RATS WITH AND WITHOUT REGENERATING ADRENALS GIVEN WATER TO DRINK

Group	Treatment	Plasma corticosterone μg/100 ml.	Adrenal corticosterone	
			μg/gm.	μg/rat
Intact Adrenals	Unstressed	5.2	5.7	0.23
	Stressed	61.5	64.5	2.73
2 Regenerating Adrenals	Unstressed	6.6	12.9	0.48
	Stressed	41.0	48.0	1.75
1 Regenerating Adrenal	Unstressed	6.5	26.4	0.53
	Stressed	25.0	43.7	0.83

volume in vacuo to about 1 ml. This was chromatographed on Whatman #1 filter paper in the E_2b system (10) for 4 hours. The zones absorbing ultraviolet light and having the same Rf as the corticosterone standards were cut out and extracted 4 times with methanol and reduced in vacuo to 10 ml. Three 0.5 ml. aliquots of each extract, including one paper blank were taken for fluorometric measurement using a modification of the Zenker and Bernstein procedure (11) for the determination of corticosterone. Three other 0.5 ml. aliquots of each extract were oxidized by the wet oxidation procedure of Van Slyke-Folch (12), and the CO_2 precipitated as barium carbonate. These samples were counted using a D-47 gas flow geiger tube for sufficient time so that the probable error (13) was less than 1%. Corrections for self-absorption was made with samples of $BaC^{14}O_3$ prepared from standard $Na_2C^{14}O_3$ obtained from the National Bureau of Standards. Using the isotopic dilution calculations described by Peterson (14), the new specific activities in d/m per μg were determined and the amounts of corticosterone originally present in the plasma and adrenal pools were determined. Triplicate aliquots of the same pool carried through the above procedures agree to within ±4%. The series of experiments were repeated and essentially the same results were found.

RESULTS

The results of the first experiment are presented in Table 1. In each of the 3 different groups of animals, the levels of circulating corticosterone were similar under resting conditions, but in response to stress, the animals bearing regenerating adrenals had circulating levels from 40% to 67% of those with intact glands. In rats with intact adrenals, plasma levels seemed to be a direct reflection of the amount of corticosterone in the glands under both resting and stressed conditions, but in the groups with regenerating

TABLE 2. CORTICOSTERONE CONTENT OF PERIPHERAL PLASMA AND ADRENAL TISSUE IN RATS WITH AND WITHOUT REGENERATING ADRENALS GIVEN 1% NaCl TO DRINK

Group	Treatment	Plasma corticosterone μg/100 ml.	Adrenal corticsoterone	
			μg/gm.	μg/rat
Intact Adrenals	Unstressed	4.6	3.2	0.12
	Stressed	69.2	49.5	1.78
2 Regenerating Adrenals	Unstressed	5.3	16.9	0.37
	Stressed	29.8	45.8	0.93
1 Regenerating Adrenal	Unstressed	2.7	20.6	0.42
	Stressed	21.1	54.0	1.02

adrenal tissue this relationship did not seem to hold under both circumstances. Thus, under resting conditions, the corticosterone content of the regenerating glands was 2 to 4 times greater than would have been anticipated from the plasma value, while in response to stress the plasma level rose relatively more than the increase in adrenal content of corticosterone.

The circulating levels of corticosterone in rats with intact or 2 regenerating adrenals drinking 1% saline were not appreciably different under either resting or stressed conditions from those drinking water (Table 2). In animals with 1 regenerating gland, however, the resting plasma corticosterone value was about one half that observed in rats given water to drink. The adrenal tissue of control animals under resting and stressed conditions contained less corticosterone than in similar rats allowed water to drink. The ability of regenerating glands to secrete corticosterone in

TABLE 3. CORTICOSTERONE CONTENT OF PERIPHERAL PLASMA AND ADRENAL TISSUE IN RATS WITH AND WITHOUT REGENERATING ADRENALS, UNINEPHRECTOMIZED, AND GIVEN 1% SALINE TO DRINK

Group	Treatment	Plasma corticosterone μg/100 ml.	Adrenal corticosterone	
			μg/gm.	μg/rat
Intact Adrenals	Unstressed	5.1	4.4	0.15
	Stressed	41.3	42.2	1.45
2 Regenerating Adrenals	Unstressed	4.7	23.9	0.63
	Stressed	37.4	48.5	1.20
1 Regenerating Adrenal	Unstressed	4.4	19.6	0.37
	Stressed	22.9	36.9	0.72

response to stress under salt load was again less than that of intact adrenals as reflected by the lower plasma corticosterone levels, although the concentration of corticosterone in the regenerated tissue was about the same as in the first experiment.

The data in Table 3 shows that reduction in renal mass and imposition of a salt load in control animals with intact adrenals failed to influence the resting plasma levels of corticosterone, but under stress the plasma and adrenal content of corticosterone was less than that observed in preceding experiments. The levels of hormone in the plasma and glandular tissue of stressed animals bearing two regenerating adrenals shows their response was about the same as similar animals in experiments 1 and 2 and the control rats in this experiment. However, animals bearing one regenerating adrenal had plasma and adrenal corticosterone levels after stress which were about 60% of those with 2 regenerating glands. It is again noteworthy that while plasma corticosterone levels were about normal under resting conditions, the regenerating glandular tissue concentration and total content of corticosterone was considerably higher than in control animals.

DISCUSSION

Although many groups of investigators (15–17) have reported the corticosterone content of peripheral plasma in the rat to be less than 20 μg/100 ml. under resting conditions, only Fortier and coworkers (18–20) and Guillemin *et al.* (21) found corrected values from 3 to 8 and from 4.5 to 6.5 μg/100 ml. plasma, respectively. The present experiments in which rigidly controlled conditions were used for the collection of plasma in the "resting" state, and corticosterone was determined by isotopic dilution, have yielded values ranging from 2.7 to 6.6 μg corticosterone per 100 ml. of plasma. We believe that these values truly represent the level of corticosterone in the plasma of unstressed rats and that finding essentially the same levels in all such groups supports this contention.

Masson, Koritz and Peron (3) showed that the concentration of corticosterone in adrenal vein blood of uninephrectomized animals bearing a regenerating adrenal was about one third that of controls (130 μg/100 ml. vs. 370 μg/100 ml.), and our data shows that the concentration of corticosterone in peripheral plasma of similar rats bearing a regenerating adrenal and stressed (as their rats must have been by the operative procedure) was slightly over half (22.9 μg/100 ml.) that of control animals (41.3 μg/100 ml.). Both observations strongly indicate that less corticosterone is being secreted under stress by the regenerating gland than by the normal gland. *In vitro* studies with regenerating adrenals by Masson *et al.* (3) and Macchi and Wyman (22) also showed that glands regenerating for this period of time have a decreased capacity to secrete corticosterone in response to ACTH stimulation. The lower plasma levels of corticosterone in the present *in vivo* studies could be explained by decreased blood flow from regenerating tissue, but this seems unlikely, as Pellegrino and Brogi (5) have shown that there is only a 15 percent reduction in blood flow through the regenerating gland at 21 days. In all experiments the total glandular content of corticosterone in stressed animals bearing regenerating glands was less than in both adrenals of stressed control rats. Thus, decreased liberation of corticosterone by regenerating adrenal tissue, as reflected by adrenal venous and systemic plasma levels, may be related to the finding that such glands have less corticosterone available for secretion.

Holzbauer (1) has shown an almost constant one to one relationship between the hormone concentration of plasma and adrenal glands when expressed as μg/100 ml. of plasma and μg/gm. of adrenal tissue. This relationship was found to be essentially true in 2 of the 3 experiments in animals with intact adrenals in the present study. On the other hand, animals bearing regenerating glands did not show this relationship. Under resting conditions the corticosterone concentration of regenerating glands was considerably more than in normal adrenals. The higher values reported

by Holzbauer (23) for unstressed intact adrenals, 23 ± 2.7 μg/gm., as compared to our values, from 3.2 to 5.7 μg/gm. may be due to the slight stress incurred by her practice of killing the animals in the same room rather than in an isolated area as we have done. The higher values for corticosterone in the adrenals of stressed rats which this investigator also reported (21) may be accounted for either by a greater degree of stress or by the inclusion of more polar compounds with corticosterone in the determination of this steroid.

The present data substantiates the view previously expressed (7, 25) that even under non-stressed conditions regenerating adrenal cortical tissue is highly active. This is probably a response of the morphologically regenerating tissue to the elevated endogenous ACTH secretion induced by the post-enucleation period of adrenal insufficiency (7). The previously observed (26) decrease in cholesterol and ascorbic acid concentration of regenerating adrenal tissue inferred such a functional state, since a decrease in these adrenal components has long been used as an index of increased functional activity (27). The decreased cholesterol content of regenerating glands may well result from increased conversion of this compound into corticosterone as suggested by their reciprocal relationship here and under conditions of acute ACTH stimulation in normal rats (28). On the other hand, the lower concentration of cholesterol in regenerating adrenals could serve as the limiting factor in corticosterone synthesis by regenerating adrenals, and together with the smaller size of the regenerated adrenal at 21 days, account for the decreased capacity of such glands to elevate systemic plasma corticosterone to the same extent as intact adrenals in response to stress.

Since corticosterone is the major steroid secreted by the rat adrenal, it was reasonable to assume that the hypertensive syndrome which occurs during adrenal regeneration might be related to the secretion of corticosterone by the regenerating tissue. It has been shown that administration of large quantities of corticosterone to adrenalectomized, uninephrectomized, salt-treated rats can produce elevation in blood pressure and morphological changes which are similar to those occurring during adrenal regeneration in the salt-treated, uninephrectomized rat (29). The present experiments indicate that under non-stress conditions, peripheral plasma levels of corticosterone in control animals and those bearing 21-day regenerating adrenals are the same, but under stress the levels attained in rats with regenerated adrenals were always less than in rats with intact glands. This finding makes it difficult to ascribe adrenal-regeneration hypertension simply to the blood levels of corticosterone. In view of the high corticosterone content of regenerating adrenals under non-stressed conditions, it is possible that this steroid is transformed into another compound, which was not measured in either the gland or the plasma. Such a postulated

compound might explain the role of the regenerating adrenal in the pathogenesis of this form of experimental hypertension, but these experiments offer no evidence for or against this hypothesis.

Acknowledgment

The authors wish to thank Mrs. Anne B. Upton for her skillful technical assistance.

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FURTHER STUDIES ON THE SEX DIFFERENCE IN 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY OF RAT LIVERS[1]

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ABSTRACT

Conditions have been established for quantitative assay of rat liver 3β-hydroxysteroid dehydrogenase activity in order to investigate further the difference previously demonstrated (1) in the ratio obtained of epiandrosterone/androsterone, depending on the sex of the animal from which the liver was taken. Livers of male rats have been shown to demonstrate more activity than livers of female rats, whether assays were made using crude homogenates, fractions obtained by differential centrifugation, or acetone-dried preparations. Data were also obtained indicating that rat liver contains separable 3β-hydroxysteroid dehydrogenase activities associated with particulate and soluble material, and the sex difference lies in a greater activity of the 3β-hydroxysteroid dehydrogenase associated with the particulate matter. The activity of the enzyme associated with the particulate matter can be increased in livers of female rats by administration of testosterone propionate.

IN AN earlier paper (1), data were presented demonstrating a sex difference in the reduction of the 3-keto group of androstane-3,17-dione by rat liver homogenates. Homogenates of livers from male and female rats yielded the same products, androsterone (3α-hydroxyandrostan-17-one) and epiandrosterone (3β-hydroxyandrostan-17-one) and the total quantities of reduced products were the same, but the ratio of 3β- to 3α-hydroxylated products depended upon the sex of the animal from which the liver was obtained.

This phenomenon has been investigated further, to find out wherein the sex difference lies, preliminary to elucidating the mechanism by which androgen operates to create the difference. Data will be presented in this paper showing that: (1) the higher ratio of epiandrosterone/androsterone obtained with crude homogenates of livers of male rats reported in the first reference (1) was a reflection of a greater amount of 3β-hydroxysteroid dehydrogenase activity in livers of male rats. An assay procedure for 3β-hydroxysteroid dehydrogenase has been standardized, based on the rate of production of epiandrosterone. (2) Using this assay, the sex difference could be demonstrated in cell fractions obtained by differential

Received November 4, 1960.

[1] Supported by United States Public Health Service Grant A 1752.

[2] Senior Research Fellowship SF-42 of the United States Public Health Service.

centrifugation of sucrose homogenates and in acetone-dried preparations of rat livers as well as in crude homogenates. Therefore, such preparations were suitable for further studies of the factors involved in the sex difference. (3) Studies with fractions obtained by differential centrifugation of sucrose homogenates, and even more strikingly, studies with acetone-dried preparations, gave data indicating that there were separate 3β-hydroxysteroid dehydrogenase activities associated with soluble and particulate cell fractions and the sex difference manifests itself in the greater activity of the particulate enzyme in the livers of male rats. The activity of particulate enzyme can be increased in female rats by testosterone propionate administration. (4) Evidence has been obtained suggesting that this greater activity may reflect the presence of more enzyme or an effect of testosterone in overcoming an inhibition of the particulate enzyme.

METHODS

Unless otherwise specified, incubation and analytical procedures were carried out as described in the previous paper (1). A 50% homogenate is prepared by homogenizing 1 gm. of liver in 1 ml. of liver homogenizing fluid, yielding 1.5 ml. of homogenate.

Losses of epiandrosterone during extraction and the analytical procedure were determined by recovery experiments with each type of enzyme preparation, and corrections made. From 50% homogenates, recovery after extraction and a single chromatogram

TABLE 1. INFLUENCE OF ANDROSTANE-3,17-DIONE CONCENTRATION ON AMOUNTS OF ANDROSTERONE AND EPIANDROSTERONE FORMED DURING INCUBATION WITH RAT LIVER HOMOGENATES

Sex of rat	Tissue conc. (mg.N/1.5 ml.)	Substr. conc. (μg/1.5 ml.)	Products (μg)	
			Androsterone (3 Alpha)	Epiandrosterone (3 Beta)
M	24.0	500	trace	252
		1000	213	537
		2000	445	709
		3000	445	676
M	21.7	2000	584	992
		3000	599	1061
		4000	587	1043
	11.5	2000	291	567
		3000	288	539
		4000	301	590
	5.2	2000	156	277
		3000	169	170
		4000	186	236
F	20.4	500	215	14
		1000	400	72
		2000	1150	116
		3000	1445	123
F	18.6	2000	855	219
		3000	1150	431
		4000	1030	443
		5000	1040	235

was 76%. Recovery from acetone powder suspensions was 80%, and from acetone powder extracts was 91%. It was possible to recover 93% of epiandrosterone added to 20% (w/v) sucrose homogenates and to cell fractions obtained from these. Each additional chromatogram resulted in the loss of an additional 15% of the epiandrosterone.

Acetone-dried liver preparations were prepared in the cold (7°). Livers were homogenized with 10 volumes of cold acetone in a Waring blendor 2 times, for 30–60 seconds each time. Acetone was removed by filtration, followed by drying of the acetone-insoluble material at room temperature for 1–2 hours, and evacuation in a vacuum desiccator over P_2O_5 at 4°. The material was stored at −15°. Extracts of acetone-dried preparations were made by suspending the powder in 10 volumes of 0.05 M phosphate buffer, pH 7.4, and stirring for 15 minutes in the cold. Centrifugation was done at −3°, in a Lourdes centrifuge, at approximately 13,000 g.

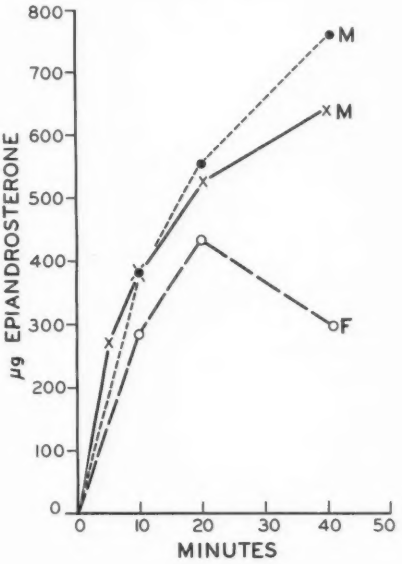

FIG. 1. Rates of production of epiandrosterone from androstane-3,17-dione by 50% (w/v) homogenates of livers from male (M) rats and female (F) rats. (Conditions of incubation: 1.0 gm. liver in 1.5 ml. homogenate in liver homogenizing fluid, 3 mg. androstanedione, 3 mg. DPNH, 0.06 ml. propylene glycol, 37° C, air.)

Cellular fractions were prepared from a 20% (w/v) homogenate of liver made in a Potter-Elvehjem all-glass homogenizer, in a medium containing 0.25 M sucrose, 0.001 M ethylenediaminetetracetic acid (EDTA) and 0.01 M $NaHCO_3$, pH 7.43. Thereafter, a modification of the Schneider and Hogeboom (2) differential centrifugation procedure was used. Four fractions, three sediments and a supernatant solution (IV) were obtained by successive sedimentation at 680 g for 10 minutes (I), 11,500 g for 10 minutes (II), and 105,000 g for 45 minutes (III). (See Figure 2.)

Cell fractions and those obtained from acetone-dried preparations were suspended for assay in a volume of 0.05 M phosphate buffer (pH 7.4) such that the equivalent of 1 gm. of fresh liver was contained in 1.5 ml. of suspension.

In all experiments except those described in Table 1 and Figure 1 substrate concentration was 3 mg. androstane-3,17-dione per beaker. Co-factors were added at a concentration of 3 mg. per beaker. Diphosphopyridine nucleotide (DPN) was added in the experiments reported in Table 1 and Figure 1. Reduced DPN (DPNH) was used in all other experiments.

EXPERIMENTAL

Establishment of Standard Assay Procedure: Under the conditions of incubation, the oxidation of epiandrosterone to androstanedione gave amounts of product too small to be measured, even at pH 8.6–9.0 and therefore the enzymatic activity had to be measured by the reduction of androstanedione to epiandrosterone. In all the preparations assayed, the presence of 3α-hydroxysteroid dehydrogenase made it necessary to determine substrate concentrations for maximum rates with both enzymes. In Table 1 are shown data obtained from experiments in which homogenates of male and female rat livers, equivalent to 1 gm. of tissue, were incubated with amounts of substrate varying from 500 μg to 5 mg. As the substrate concentration increased, the amounts of epiandrosterone and androsterone obtained from incubations with homogenates of male livers both increased. With homogenates of livers of female rats, it was possible to increase slightly the yield of epiandrosterone, but never to the levels obtained with homogenates of livers from male rats. Studies with concentrations of substrate up to 5 mg., showed that the substrate concentration necessary for maximum rates with both enzymes was about 3 mg./1.5 ml. It was also shown, in the experiment with several concentrations of male rat liver, that the higher levels of substrate were not inhibitory at lower tissue concentrations.

Crude homogenates of livers of male and female rats were incubated with optimal concentration of substrate for varying time intervals. The results of typical experiments, shown in Figure 1, demonstrated that although the rate began to diminish after 5 minutes, approximate linearity persisted for 20 minutes. Since, as Table 1 shows, there was no significant decrease in formation of products when the concentration of substrate was 2 mg./1.5 ml. instead of 3 mg./1.5 ml., decrease in substrate concentration during incubation did not seem to be the rate-limiting factor. Addition of larger amounts of DPN, the use of triphosphopyridine nucleotide (TPN) instead of DPN, homogenizing the liver with heated extract made from another homogenate of liver, and carrying out the incubation with N_2 as gas phase instead of air did not prolong the linear portion of the curve and had no effect on the 3β-hydroxysteroid dehydrogenase activity. A 20 minute incubation in air was adopted for the *pro tempore* standard assay procedure. This time permitted formation of sufficient product for accurate measurement. Considering the accuracy of the measurement of the amounts of product produced at 5 minutes and 10 minutes, the departure from linearity at 20 minutes was not of serious magnitude. Assays of sucrose homogenates and of dialyzed and undialyzed acetone-powder preparations also demonstrated rates that were linear over 20 minutes. The unit of enzyme activity, used hereafter, is therefore the amount of enzyme necessary to catalyze production of 1 μg of epiandrosterone per minute during a 20 minute incubation.

Sex Difference in 3β-Hydroxysteroid Dehydrogenase Activities of Crude Homogenates: The Role of Androgen: The sex difference was reinvestigated using the quantitative determination of 3β-hydroxysteroid dehydrogenase activity instead of ratio of epiandrosterone/androsterone. The activities of crude 50% (w/v) homogenates in liver homogenizing fluid (1) are listed in Table 2. In some experiments, equal portions of livers from four animals were pooled to minimize individual variation. The data show that the influence of androgen is manifest whether units are related to weight of liver or to mg. nitrogen in the preparation. The activity in the normal male was approximately 10 times that in the normal female.

Castration of mature males lowered the 3β-hydroxysteroid dehydrogenase activity to about 70% of normal after 30 days and to about 7% of normal after 120 days. Activity in a normal male was doubled by the administration of 2.5 mg. testosterone propionate daily for 28 days.

TABLE 2. INFLUENCE OF ANDROGEN ON 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES OF 50% HOMOGENATES OF RAT LIVERS

Sex	No. rats	Treatment	Activity	
			u./mg. N	u./gm. liver
F	Pool of 4	Normal	0.12	2.8
F	Pool of 4	Normal	0.17	3.8
M	Pool of 4	Normal	1.41	31.0
M	Pool of 4	Castrate, 30 days	1.49	22.8
M	1	Castrate, 120 days	0.08	2.3
M	Pool of 4	Normal, 2.5 mg. TP×28[1]	2.56	54.0
M	Pool of 4	Castrate, 1.6 mg. TP×28[2]	1.04	23.0
F	1	OvariX,[3] 1.1 mg. TP×14[2]	0.21	4.6
F	1	OvariX, 1.5 mg. TP×28[2]	0.75	17.6
F	1	OvariX, 1.5 mg. TP×28[2]	0.87	20.0
F	Pool of 4	OvariX, 2 mg. TP×28[1]	1.26	31.0

[1] TP = testosterone propionate, subcutaneous injection once daily.
[2] Ave. daily absorption from 75 mg. pellet, implanted 35 days p castration.
[3] OvariX = ovariectomized, T.P. administration started day after ovariectomy.

Administration to ovariectomized female rats of sufficient quantities of testosterone propionate, over a long enough period of time, elicited levels of 3β-hydroxysteroid dehydrogenase activity comparable with those found in the livers of male rats. An ovariectomized female not treated sufficiently (first in series) had a level of activity of the same order of magnitude as that of normal, untreated females.

Evidence for Particulate and Soluble 3β-Hydroxysteroid Dehydrogenases and Sex Differences: Assays of fractions obtained by differential centrifugation of sucrose homogenates indicated that there was activity in both soluble and particulate cell fractions and that the sex difference in activity was demonstrable only in the enzyme associated with particulate matter (see Fig. 2). Also, androgen influenced the distribution of the total activity

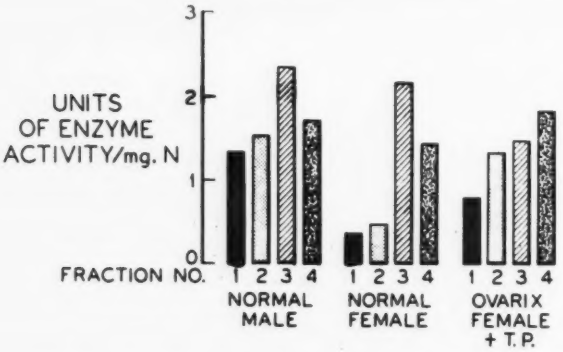

FIG. 2. Influence of androgen on 3β-hydroxysteroid dehydrogenase activities of cell fractions. (Conditions of incubation: cell fractions 1, 2, and 3 were suspended so that equivalent of 1 gm. of liver was contained in 1.5 ml. of 0.05 M phosphate buffer, pH 7.4. Supernatant fraction (4) contained equivalent of 0.27 gm. liver/1.5 ml. in 0.25 M sucrose, 0.001 M EDTA, and 0.01 M $NaHCO_3$, pH 7.43. 3 mg. androstanedione, 3 mg. DPNH, 0.06 ml. propylene glycol, 37° C, air, 20 min. incubation.) (OvariX = ovariectomized, T.P. = 2 mg. Testosterone propionate per day, injected subcutaneously in oil, for 44 days.)

between soluble and particulate fractions. Although in all experimental animals the nitrogen content of the particulate matter exceeded that of the supernate (52–72% of the total), the percentages of total 3β-hydroxysteroid dehydrogenase activities associated with the particles were: male

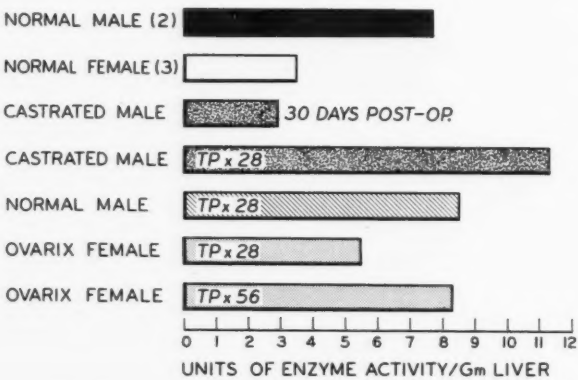

FIG. 3. Influence of androgen on 3β-hydroxysteroid dehydrogenase activites of acetone-dried preparations. (Conditions of incubation: all fractions contained the equivalent of 1 gm. of fresh liver in 1.5 ml. 0.05 M phosphate buffer, pH 7.4. 3 mg. androstanedione, 3 mg. DPNH, 0.06 ml. propylene glycol, 37° C, air, 20 min. incubation.) (Castrated male and ovariectomized females 1-2 mg. testosterone absorbed per day from a 75 mg. pellet of testosterone propionate, number of days 28 or 56. Normal male injected subcutaneously with 2.5 mg. testosterone propionate per day, for 28 days.

rats, 52–64%; female rats 35–48%; ovariectomized female rats treated with testosterone propionate, 57–63%; castrate male rats, 45–50%.

A more graded fractionation of homogenates provided particles sedimenting between 7000 and 26,360 g which had 2 to 5 times as much activity when obtained from male rats or from female rats treated with testosterone as when obtained from normal female rats.

Soluble and Insoluble 3β-Hydroxysteroid Dehydrogenases in Acetone-Dried Preparations: As shown in Figure 3, the influence of androgen was demonstrable in acetone-dried preparations as well as in fresh homogenates although the magnitude of the difference was less. Normal males and animals treated with testosterone propionate had activities of the order of

TABLE 3. COMPARISON OF 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES OF FRESH 50% HOMOGENATES OF RAT LIVER WITH ACTIVITIES OF ACETONE-DRIED POWDERS FROM SAME RAT LIVERS

Sex[1]	No. assays	Preparation	Avg. activity	
			c./mg.N (range)	c./gm. liver (range)
F	2	50% homog.	0.14 (0.12–0.17)	3.3 (2.8–3.8)
		Acetone powder	0.12 (0.08–0.17)	2.6 (2.0–3.1)
M	1	50% homog.	1.41	31.0
		Acetone powder	0.38	8.7
F, ovariX+TP	3	50% homog.	0.96 (0.75–1.26)	22.9 (17.6–31.0)
		Acetone powder	0.15 (0.13–0.17)	4.3 (4.2– 4.4)
M, normal+TP	1	50% homog.	2.56	54.0
		Acetone powder	0.30	8.5

[1] OvariX = ovariectomized, TP = treated with testosterone propionate.

2 to 3 times those of female rats or untreated castrate males, rather than 10 times. When the activities of the suspended acetone-dried powders were compared with the activities of fresh 50% homogenates from the same livers (Table 3), it appeared that much activity was lost in preparing powders from livers of male rats or from livers of rats treated with testosterone propionate, but not from livers of untreated female rats. Activity was not restored by adding the acetone-soluble material. In the acetone-dried powder preparations as well as in the homogenates made in sucrose solution, a greater proportion of activity was associated with the insoluble particulate fraction of liver from male rats compared to liver from female rats, although, as before, there was no sex difference in the distribution of nitrogen content (21–28% of the total nitrogen remaining in the extracted particulate fraction). After exhaustive extraction (4 times, with 0.05 M phosphate buffer, pH 7.4) 25–35% of the activity remained with the particles obtained from males and only 14–16% with those from females. These residual activities could not be removed by further extraction with

TABLE 4. EFFECT OF FRACTIONATION ON 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES OF RAT LIVERS: ACTIVITIES OF 20% HOMOGENATES VERSUS SUM OF ACTIVITIES OF CENTRIFUGAL FRACTIONS

Sex	No. of assays	Activities (U./gm. liver)	
		20% homog. (range)	Sum of fractions (range)
F	3	11.2 (7.1–16.0)	24.2 (16.0–29.1)
M	3	28.4 (16.9–32.6)	33.8 (22.3–40.0)
F, ovariX+TP[1]	2	19.5 (19.0–20.0)	27.8 (25.4–30.2)

[1] OvariX+TP=ovariectomized female treated with testosterone propionate.

buffer. Livers of ovariectomized female rats treated with testosterone propionate were similar in behavior to those of male rats.

Evidence for Inhibition of the Particulate Enzyme: As shown in Table 4, the sum of activities of fractions obtained by centrifugation of a 20% homogenate always exceeded the activity of the homogenate *per se*, an effect particularly striking if normal female rats were used.

A similar observation was made when the sum of activities of extracts of acetone-dried preparations plus the activity of the remaining particles was compared with activity of the initial suspension (Table 5). The increment of summed activities was again larger in female rats. It is also seen that the summed activity in the female rat liver acetone-dried powder was higher than that of 50% fresh homogenates of the same livers, and the summed activities for normal male rats and animals treated with testosterone were closer to those seen in 50% homogenates (see Table 3) than the corresponding activities of whole suspensions.

Suspensions of acetone-dried preparations of livers of both male and female rats were assayed at various concentrations, and both showed increases in units per mg. N as the suspension was diluted. For the female, a suspension containing one-seventh as much N was 12 times as active per mg. N as the concentrated suspension. For the male, a suspension with one-eighth as much N was 5 times as active per mg. N as the concentrated suspension. In two experiments, insoluble material from acetone-dried

TABLE 5. EFFECT OF EXTRACTION ON 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN ACETONE-DRIED PREPARATIONS: ACTIVITIES OF WHOLE SUSPENSION VERSUS SUM OF ACTIVITIES OF EXTRACT AND EXTRACTED PARTICLES

Sex	No. of assays	Activity—U./gm. Liver	
		Whole susp.[2] (range)	Sum of extr.+Part.[3] (range)
F, normal	1	2.0	10.3
M, normal	1	8.7	17.9
F, ovariX+TP[1] (4 weeks)	2	4.3 (4.2–4.4)	7.8 (5.6–10.0)
F, ovariX+TP (8 weeks)	1	8.3	23.0
M, normal+TP	1	8.5	20.3

[1] OvariX=ovariectomized, +TP=treated with testosterone propionate.
[2] 1 gm. powder to 10 ml. in .05M phosphate buffer, pH 7.4.
[3] Sum of activities of 4–5 extracts plus activity of extracted particles.

preparations from livers of male rats, which had been extracted 4 times, with buffer, still showed very striking increases in activity on dilution.

These effects could be due to the presence of some endogenous inhibitor, or to partial unavailability of the enzyme in more concentrated suspensions. An inhibitor was sought in homogenates of liver from a female rat by incubating a mixture of equal aliquots of 50% homogenates of livers of female and male rats. The homogenate obtained from the liver of the female rat did not inhibit the activity of that from the male rat, and actually 134% of the expected amount of epiandrosterone was obtained. Assays of dialyzed acetone-powder extracts, of diluted soluble preparations from acetone-powders, and assays made with the addition of heated extract from liver did not suggest the presence of any soluble inhibitor.

DISCUSSION

In the last few years several papers have appeared describing both quantitative and qualitative differences due to sex in liver metabolism of steroid hormones or their metabolites. Hübener *et al.* (3) reported that reduction of the 11-keto group of cortisone was greater with homogenates of liver from male rats than from females. This was followed by the demonstration of the sex difference in the orientation of the reduction products of androstane-3,17-dione (1). Forchielli, Brown-Grant and Dorfman (4) and Yates *et al.* (5), presented data showing that the rate of reduction of the Δ^4-3-ketone structure of many steroid hormones was faster in homogenates from livers of female rats than from male rats. Both groups of workers demonstrated that the increase in rate was due to greater concentration of microsomal Δ^4-5α-hydrogenase activity in livers of female rats. Yates *et al.* (5) showed further that the sex difference did not involve either the availability of reduced pyridine nucleotides, or the presence of activators in the females or inhibitors in the males. Both groups of investigators also showed that testosterone was the controlling gonadal hormonal factor; however, extra-gonadal factors, e.g., adrenal and pituitary, also were implicated. Leybold and Staudinger (6) also observed a quantitative difference, due to sex, in rates of reduction of the Δ^4-3-ketone, and presented evidence of differences in nature of end-products as well. Incubation of testosterone and androstane-3α,17β-diol with liver microsomes from males gave rise to more polar steroids, possibly 6β-hydroxylated compounds. These products were not obtained with microsomes from livers of female rats.

From the data presented in Figure 1, it can be concluded that the ratio of epiandrosterone/androsterone formed, the index used in the first paper (1), is an index whose value will vary with the substrate concentration, when substrate concentration is less than optimal. However, at levels of substrate sufficient to saturate both 3α-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase, there is still a difference between male and female rat livers. There is more 3β-hydroxysteroid dehydrogenase

activity in livers of male rats than in livers of female rats, as measured by an assay procedure based on the rate of production of epiandrosterone from androstanedione.

Furthermore, data presented indicate that the increased activity is found in tightly-bound particulate 3β-hydroxysteroid dehydrogenase activity. Livers of both sexes demonstrate approximately equal activities of corresponding soluble enzymes. The greater particulate activity was shown to depend on presence of testosterone.

There are at least six ways in which testosterone may influence 3β-hydroxysteroid dehydrogenase activity:

1. Testosterone may stimulate apoenzyme synthesis as part of a generalized anabolic effect.

2. Since androstanedione arises from testosterone, enzyme synthesis may be stimulated as an adaptive enzyme phenomenon, although it requires a longer period of administration of "inducer" than the other reported instances of adaptation (7, 8).

3. If one assumes that the particulate enzyme and the soluble enzyme are the same, testosterone may act to control the rate of transfer of the enzyme in and out of the particle. Adams (9) suggests that androgen influences the distribution of mouse liver catalase between particulate and extra-particulate fractions by governing the "permeability" of the granule membrane. He observed this effect after a single administration of testosterone or methyltestosterone.

4. Rather than influencing the protein portion of the enzyme, testosterone may control the availability of a co-factor or affect a more remote step of enzymatic control. There is no evidence yet that testosterone acts directly; the effect may be mediated through some other gland, such as the adrenal or the pituitary.

5. The data indicate that a possible mechanism by which testosterone acts is by promoting removal of a factor inhibiting the particulate enzyme. The increased activity following extraction or dilution of homogenates of livers from female rats or of acetone-dried powder suspensions was of much greater magnitude than that observed when liver preparations from males were treated in the same manner. The apparent loss of activity when acetone-dried powders were made of liver of male rats might be due to concentration of an inhibitor.

6. Still another possible role for testosterone is to alter the availability of active sites on the protein molecule, perhaps by changes in the molecular configuration of the enzyme or by changes in the degree of aggregation of protein molecules.

Obviously, before testing the first three hypotheses, one must purify the soluble and particulate enzymes, and determine whether they are the same enzyme in different compartments or actually different enzymes. Tompkins (10) has purified 3α-hydroxysteroid dehydrogenase from rat liver and

studied its properties. Hurlock and Talalay (11) purified 3α-hydroxysteroid dehydrogenase from 105,000 g supernate of a 0.25 M sucrose homogenate of rat liver and reported that the preparation contained some 3β-hydroxysteroid dehydrogenase activity. Purification of a particulate 3β-hydroxysteroid dehydrogenase has not been reported. Hamm, Kochakian, and Carroll (12) showed 3β-hydroxysteroid dehydrogenase activities in both supernatant and particulate fractions from the livers of male guinea pigs. Endahl, Kochakian, and Hamm (13) have since then shown that the particulate enzyme is DPN-specific and the soluble enzyme is TPN-specific.

Hypotheses 4, 5, and 6 are also under investigation.

Addendum

Since this paper was submitted to press, Dr. Eisler has advised us that the pellets used were of testosterone, not of testosterone propionate. Therefore in Figure 3 and Tables 2, 3, 4, and 5, all animals treated with pellets were treated with testosterone. Those treated by injection received testosterone propionate, as indicated in the text.

Acknowledgments

1. The authors wish to thank Mrs. Barbara Miller and Miss Carol Wachtel for their technical assistance.

2. We are greatly indebted to Dr. A. D. Odell of General Mills, Inc., Drs. Preston Perlman and Milton Eisler of Schering Corporation, and Drs. A. Zaffaroni and H. Ringold of Syntex, S.A., for generous gifts of steroid hormones.

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DIETARY SENSITIZATION FOR PARATHYROID HORMONE[1]

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ABSTRACT

Sensitization to parathyroid hormone by the oral administration of calcium acetate, sodium phosphate, and corn oil was studied comparatively in the rat.

Calcium acetate greatly faciliates the induction by parathyroid extract of calcification in the cardiovascular system, skeletal musculature, and renal cortex, as well as the production of osteitis fibrosa, particularly if, at the same time, the intake of fat is augmented. Sodium phosphate likewise increases the activity of parathyroid hormone, though in equimolecular amounts this salt is less effective than calcium acetate; moreover, sodium phosphate—unlike calcium acetate—changes the quality of the resulting morbid lesions in that the cardiac calcification is accompanied by suppuration, and the nephrocalcinosis shifts to the corticomedullary junction. The effects of combined treatment with parathyroid hormone and sodium phosphate are likewise aggravated by orally administered fat-supplements.

Attention is called to the fact that the same sensitizing agents (calcium acetate, sodium phosphate, dietary fat-supplements) that are effective in increasing the actions of parathyroid hormone also proved to be potent sensitizers for the induction of cardiovascular lesions by certain vitamin-D derivatives and corticoids.

THE oral administration of sodium phosphate not only increases the actions of parathyroid hormone, but qualitatively changes them. Normally, excessive amounts of this hormone produce cortical nephrocalinosis and ectopic calcium deposition in the cardiovascular system of the rat. These changes are not merely aggravated by concurrent sodium phosphate treatment, but are so altered that calcium deposition in the kidney tends to predominate at the corticomedullary junction, while the cardiac lesions assume a definitely suppurating character (1). In all these respects, sodium phosphate modifies the effects of dihydrotachysterol intoxication in the same manner (2, 3). Oral administration of corn oil

Received November 10, 1960.

[1] This work was made possible by Grants Nos. A-1641(C2) and H-3688(C3) from the National Institutes of Health, U.S.P.H., and by a grant from The Dept. Health and Welfare, Quebec, Canada. The authors are particularly grateful to Dr. W. M. Mikulashek (International Clinical Research Department, The Lilly Research Laboratories) for general supplies of "Para-thor-mone."

[2] Fellow of The Rockefeller Foundation.

TABLE 1. DIETARY SENSITIZATION FOR PARATHYROID HORMONE
(First experiment)

Group	Treatment*	Cardiac lesions (Scale: 0–3)	Nephrocalcinosis		Mortality (%)
			Degree (Scale: 0–3)	Type	
1	None	0	0.6 ± 0.20	Cortical	0
2	Na_2HPO_4	0	0.8 ± 0.20	Corticomedullary	0
3	Ca-acetate	1.0 ± 0.30	1.8 ± 0.20	Cortical	0
4	Corn oil	0	1.0 ± 0.23	Cortical	0
5	Corn oil + Na_2HPO_4	0.3 ± 0.10	2.0 ± 0.15	Corticomedullary	30
6	Corn oil + Ca-acetate	2.4 ± 0.33	2.4 ± 0.33	Cortical	90

* In addition to the treatments listed in this column, the rats of all groups received 50 U.S.P. units of *parathyroid horomone* twice daily, as indicated in the text.

greatly augments the toxic actions of dihydrotachysterol overdosage, but does not change their character (4).

In this communication, we wish to report upon experiments showing that orally administered calcium acetate, unlike sodium phosphate, increases the effectiveness of parathyroid hormone without changing the quality of the response and that corn-oil supplements further augment this increase.

MATERIALS

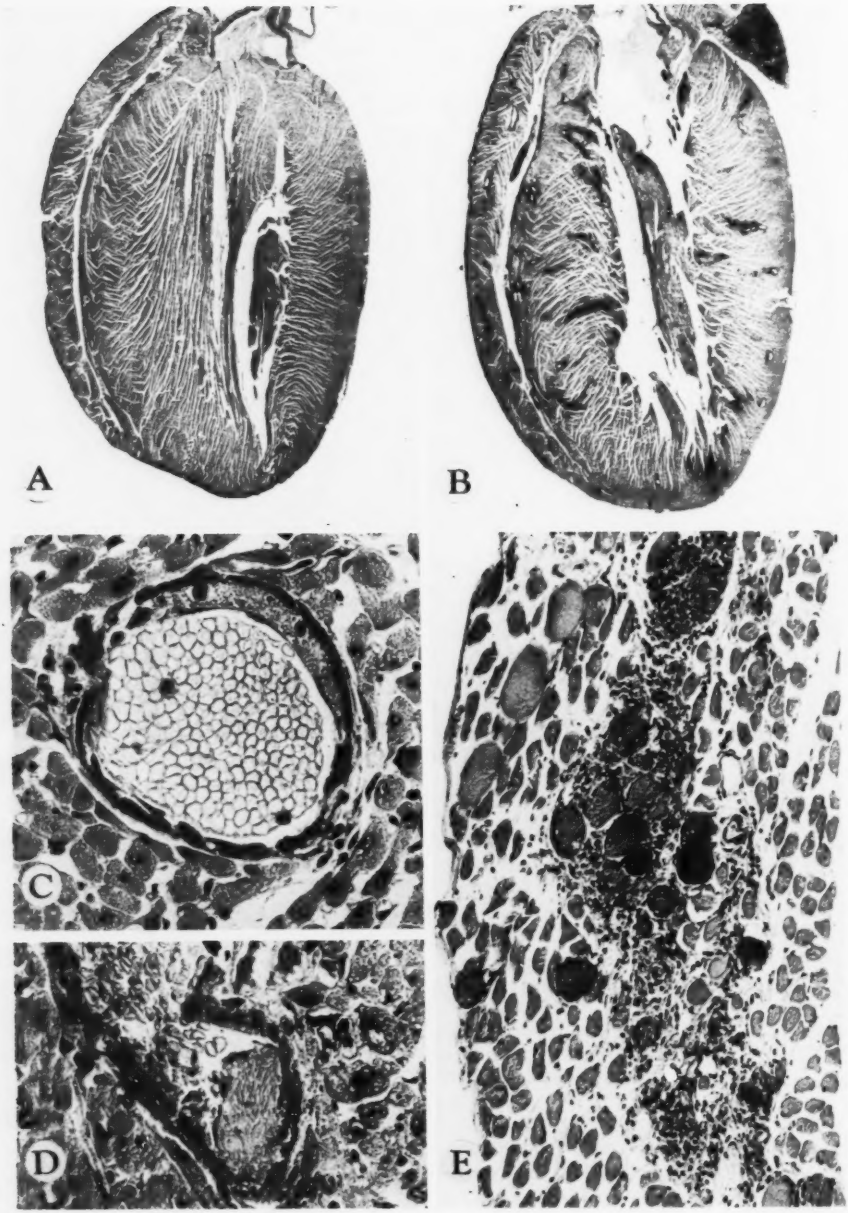
Eighty female Holtzman rats, with a mean initial body weight of 100 gm. (range: 95–106 gm.), were subdivided into eight equal groups and treated as indicated in Tables 1 and 2. In the first experiment (Table 1), parathyroid extract (Lilly) was injected subcutaneously at the dose of 50 U.S.P. units in 0.5 ml. of water, twice daily; Na_2HPO_4 and calcium acetate were given in the amount of 1 mM in 2 ml. of water by stomach tube, twice daily, while corn oil was administered by the same route at the dose of 1 ml., likewise twice daily. The experiment was terminated after three days.

In the second experiment (Table 2), treatment with acetate and corn oil was the same, but in order to permit more prolonged survival the individual dose of parathyroid extract was reduced to 30 U.S.P. units in 0.3 ml. of water. The experiment was terminated on the eighth day.

In both experiments, the rats were maintained exclusively on Purina fox chow (Purina Co. of Canada) and tap water. All survivors were killed with chloroform at the end of the observation period and the hearts, quadriceps femoris muscles, and kidneys

TABLE 2. DIETARY SENSITIZATION FOR PARATHYROID HORMONE
(Second experiment)

Group	Treatment	Cardiac lesions (Scale: 0–3)	Nephrocalcinosis		Osteitis fibrosa (Scale: 0–3)	Mortality (%)
			Degree (Scale: 0–3)	Type		
1	Paraffin oil + Ca-acetate + Parathyroid hormone	0.5 ± 0.15	0.4 ± 0.17	Cortical	1.2 ± 0.20	20
2	Corn oil + Ca-acetate + Parathyroid hormone	2.5 ± 0.20	2.7 ± 0.20	Cortical	2.8 ± 0.23	80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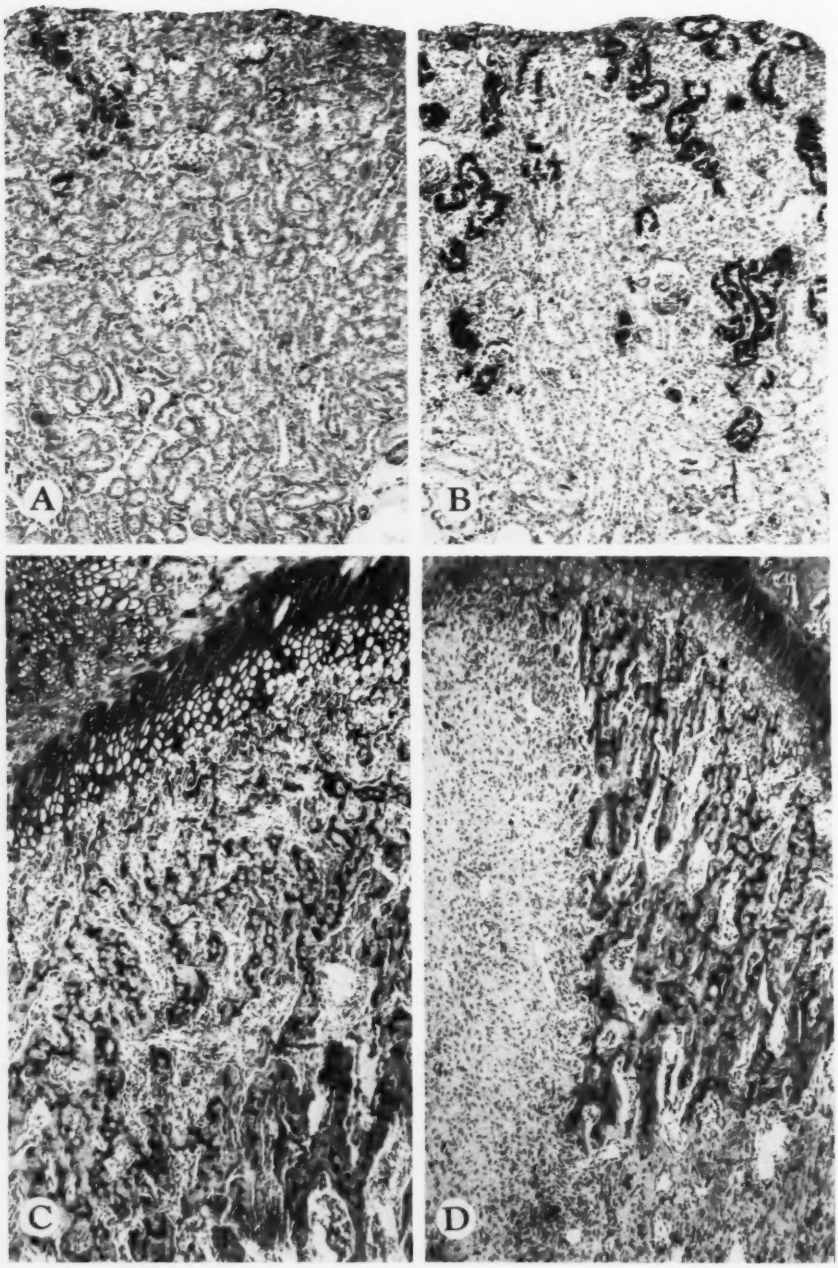
were fixed in alcohol-formol for the subsequent histochemical demonstration of calcium with the von Kóssa method. In addition, one femur was fixed and decalcified, in Susa solution saturated with picric acid, and subsequently stained with the PAS procedure and hematoxylin-phloxine. The organ lesions were graded in terms of an arbitrary scale in which 1 designates barely detectable, 2 marked, and 3 most severe, lesions. The means of these readings (with standard errors) and the percentual mortality rates are listed in the tables.

RESULTS

First experiment (*Table 1*).—The first experimental series showed that, when given in equimolecular amounts, calcium acetate is much more effective than Na_2HPO_4 in aggravating the syndrome of parathyroid-hormone intoxication. Indeed, in this short-term experiment, parathyroid extract produced marked *cardiac lesions*, only when given in combination with calcium acetate. The effect of this combined treatment (Group 3) was slightly, but significantly ($P < 0.05$), aggravated by corn oil (Group 6). Whether or not oil-supplements were given, the lesions consisted of virtually pure calcification, sometimes accompanied by the subintimal deposition of fibrinoid material but never by suppuration (Fig. 1A, B, C, and D). Conversely, the lesions induced by parathyroid extract in combination with Na_2HPO_4+corn oil (Group 5), though very mild, were associated with suppuration.

The *nephrocalcinosis* produced by parathyroid hormone was augmented both by Na_2HPO_4 and by calcium acetate; but while the former salt induced calcium deposition almost exclusively at the corticomedullary junction, parathyroid hormone alone or in combination with calcium acetate produced a predominantly cortical nephrocalcinosis. The apparent aggravation of the nephrocalcinosis by corn oil was statistically significant only in the rats receiving parathyroid hormone+Na_2HPO_4 (Group 2 compared with Group 5, $P < 0.01$), although the mean values were slightly higher in all the fat-treated groups than in the corresponding controls. Since almost all the rats treated with parathyroid hormone in combination with corn oil+calcium acetate died during the first two days of the experi-

FIG. 1. *A.*—Essentially normal heart of rat (second experiment, Group 1) treated with paraffin oil+calcium acetate+parathyroid hormone (von Kóssa ×8). *B.*—Numerous foci of calcification throughout the myocardium and its vessels in a rat (second experiment, Group 2) treated with corn oil+calcium acetate+parathyroid hormone (von Kóssa ×8). *C. and D.*—Higher magnification of cross sections through two small arteries of the heart shown in Fig. B. There are calcium deposits (black) in the arterial wall and subintimal accumulations of fibrinoid material (gray) in the form of crescents or cushions (von Kóssa ×590). *E.*—Typical skeletal lesion in the quadriceps of a rat from Group 2 of the second experiment. Swelling, homogenization, and calcification of certain muscle fibers, with inflammation in the surrounding area (von Kóssa ×180).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ment, the survival period might not have been sufficiently long to permit more intense calcium deposition (Fig. 2A and B).

Osteitis fibrosa was more pronounced in all groups treated with corn oil than in the corresponding controls, but the lesions were not sufficiently pronounced to permit clear-cut quantitative evaluation.

Pronounced *calcification, necrosis, and inflammation in the skeletal musculature* (Fig. 1 E) was evident only in the rats treated with parathyroid extract+calcium acetate+corn oil (Group 6).

Interestingly, in many animals receiving Na_2HPO_4 or calcium acetate, in addition to corn oil+parathyroid hormone, a pronounced *lipemia* developed, which imparted a milky appearance to the blood.

Second experiment (*Table 2*).—In order to obtain more definite proof of the sensitizing effect of corn oil a second experiment was performed, in which the control rats were given paraffin oil, and the experimental animals received corn oil, in addition to calcium acetate+parathyroid hormone. Otherwise, the conditions of experimentation were the same as in the first experiment, except that—as explained in the Materials section—the individual dose of parathyroid hormone was decreased from 50 to 30 U.S.P. units to permit longer survival.

Here, corn oil significantly aggravated the cardiac, muscular, renal, and skeletal lesions, as well as the mortality, produced by parathyroid hormone+calcium acetate. In both groups, the *cardiovascular lesions* consisted of pure calcium deposition without suppuration, and the *nephrocalcinosis* was limited to the cortex. In this more chronic experiment, the *muscular lesions* were particularly severe and it became obvious that these as well as the *osteitis fibrosa* induced by parathyroid hormone are likewise greatly intensified by the fat-supplements (Fig. 2 C and D). A striking degree of *lipemia* was evident in virtually all the animals treated with corn oil +parathyroid extract. This unexpected effect of the hormone upon lipemia will be the subject of a more detailed chemical study; let us point out here, however, that in numerous experiments—performed for different purposes but under similar conditions—corn oil did not produce any comparable degree of lipemia in otherwise untreated control rats.

←

FIG. 2. *A*.—Mild cortical nephrocalcinosis in a rat (Group 1 of second experiment) treated with paraffin oil+calcium acetate+parathyroid hormone (von Kóssa ×120). *B*.—Intense cortical nephrocalcinosis in a rat (Group 2 of second experiment), following treatment with corn oil+calcium acetate+parathyroid hormone (von Kóssa ×120). *C*.—Mild osteitis fibrosa in the subepiphyseal layer of the femur of the rat whose kidney is shown in *A* (PAS×85). *D*.—Particularly pronounced osteitis fibrosa in the corresponding region of the femur of the rat whose kidney is shown in *B* (PAS×85).

DISCUSSION

From these observations, it appears that the effect of parathyroid hormone can be significantly increased, not only by the oral administration of calcium salts or of phosphates, but also by lipid-supplements. It is noteworthy that the same food constituents that proved to be effective in sensitizing for the actions of parathyroid hormone were previously found to be particularly potent sensitizing agents for the production of cardiovascular lesions by vitamin-D derivatives (e.g., dihydrotachysterol) and by corticoids (2, 3).

It would be premature to speculate upon the mechanism through which these electrolytes and lipid-supplements exert their sensitizing action. In essence, the present experiments merely confirm that the observations made previously regarding dietary sensitization for dihydrotachysterol also apply to parathyroid hormone. It is of interest, however, that not only the intensity but even the quality of the response to this hormone can be altered by natural food constituents. Very little is known about the participation of the parathyroids in the pathogenesis of diseases other than simple primary hyperparathyroidism with osteitis fibrosa and ectopic calcification. Yet, it is possible to produce selective cutaneous calcinosis and scleroderma-like connective tissue proliferation in a predetermined skin region of parathyroid-hormone treated animals, merely by exposing it to mechanical or chemical trauma (5). In this respect, the actions of the hormone can also be duplicated by dihydrotachysterol (6).

All these observations suggest the possibility that parathyroid hormone and vitamin-D derivatives may participate in the pathogenesis of diverse, seemingly unrelated, morbid lesions in various organ systems (e.g., the heart, vessels, skeletal muscles, kidney, bone, skin), whose selective sensitivity to the potential pathogenic actions of these agents is largely dependent upon topical and systemic conditioning factors.

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THE INFLUENCE OF ESTRADIOL ON THE COMPOSITION AND RESPIRATION OF OVIDUCT TISSUE IN THE IMMATURE CHICK

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ABSTRACT

Estrogen administration to the immature fowl, to give oviduct growth rates comparable to those found in the pullet prior to the onset of egg-laying was studied. In a preliminary experiment the level of estradiol required to induce such a growth response in six-week-old chicks was found to be more than 2 mg. administered over a 10-day period. Maximal growth response was produced by 10 mg. estradiol. Two experiments are described in which two levels of estradiol giving growth responses dependent on dose level, i.e. 0.75 mg. and 1.5 mg., are compared with a level of 10 mg. giving maximal response. Oviduct tissues from these experiments were examined for their respiration rate, moisture, protein, fat, pentosenucleic acid and deoxypentosenucleic acid contents, and alkaline phosphatase activity. Moisture content of the tissue decreased with increasing hormone treatment. Fat content increased and on a fresh tissue basis so did protein content. Hypertrophy resulting from 10 mg. estradiol increased the ratio of pentosenucleic acid to deoxypentosenucleic acid, but decreased the respiration rate and alkaline phosphatase activity which were stimulated by 1.5 mg. estradiol.

THE very large increase in the oviduct weight of the immature chick caused by administration of estrogen is well known (1). The levels of estrogen necessary to produce oviduct responses dependent on dose level have been established by several workers (2, 3), who have used oviduct weight as a measure of the potency of estrogens or their antagonists. Little work has been published on the gross composition of the oviduct in relation to its size or to the level of hormone administration. Brown and Jackson (4) have examined the composition of oviducts from normal and folic acid deficient chicks treated with estrogens and found differences in the moisture content and alkaline phosphatase content of the tissues. In the rat, estrogen administration has been shown to increase the formation of nucleic acids in uterine tissues (5, 6). The association of increased alkaline phosphatase activity in the rat uterus with estrogen administration has also been described (7, 8). The respiration rate of uterine tissues from the castrated rat has been shown to be increased by estrogen treatment (9, 10), and this effect is maximal some 16 to 24 hours after estrogen administration. The authors were unable to find reference in the literature to the oxygen con-

Received November 14, 1960.

sumption of the corresponding avian organ, the oviduct, where a very different function is performed in relation to the reproductive process, so that observations on oxygen consumption of this tissue have been made in the present work. Changes in respiration rate with time following estrogen treatment are also examined. The levels of the steroid hormone, estradiol 17β, which we have employed are such as will induce rates of oviduct growth approximating to those which occur in the female chicken prior to the onset of egg-laying. Changes in the composition of the oviduct associated with these rates of growth are reported.

MATERIALS AND METHODS

In all the experiments described below, Light Sussex×Brown Leghorn female chicks were reared on a commercial chick mash for four weeks to a body weight of 250–270 gm. The birds for each experiment were weighed and randomized into groups on the basis of live weight prior to the start of hormone administration. During the period of hormone administration the groups were fed at a level of food intake (40 gm. per bird per day) compatible with reasonable growth. The increase of live weight (approximately 100 gm.) was similar in each group.

Experiment 1. Six groups each of 4 birds received the following levels of estradiol dipropionate in oil:—Group 1 received 5 intramuscular injections on alternate days of 1.0 mg. estradiol dipropionate, Group 2, 5×0.2 mg., Group 3, 5×0.1 mg., Group 4, 5×0.04 mg., Group 5, 5×0.008 mg. and Group 6, 5×0.001 mg. of estradiol dipropionate. The chicks were killed 2 days after the final injection and the oviducts carefully dissected out.

Experiments 2 and 3. Three groups of 7 birds (Exp. 2) or 9 birds (Exp. 3) 4 weeks old received the following levels of estradiol dipropionate:—Group 1 received 5 intramuscular injections on alternate days of 2.0 mg. estradiol dipropionate, Group 2, 5×0.3 mg. and Group 3, 5×0.15 mg. The birds were fed as in Experiment 1. At slaughter, samples of blood were obtained by free bleeding from the jugular vein prior to the removal of the oviducts.

Experiment 4. All chicks received 5 intramuscular injections of 2 mg. estradiol dipropionate on alternate days after being randomized at four weeks of age into 6 groups each of 3 birds. The fifth injection was timed so that it allowed for the same lapse of time (60 hours) in all groups prior to slaughter. A sixth and final injection of estradiol (2 mg.) was administered at times varying from 2 to 12 hours before slaughter.

Blood serum samples were frozen prior to drying in vacuo over P_2O_5. The complete oviducts from each bird were weighed rapidly and cut longitudinally into small segments on a Petri dish partially immersed in ice. Thin sections were cut from these segments and bulked to give a sample of approximately 100 mg. which was suspended in Robinson medium (11) for the determination of respiration rate in Barcroft differential respirometers as described by Dixon (12). The moisture content of a representative sample of the remainder was found by drying in vacuo over P_2O_5. After grinding, crude protein was determined by the Kjeldahl method and fat by chloroform extraction in drip extractors. Pentosenucleic acid (PNA) and deoxypentosenucleic acid (DNA) were determined on the dry samples using the Schneider (13) method of nucleic acid extraction followed by the Dische (14) reaction for DNA and Majbaum (15) reaction for PNA. Alkaline phosphatase activity of oviduct tissue was determined by an adaptation of the King-Armstrong (16) method to dry tissue, as described by Delory (17).

RESULTS AND DISCUSSION

It is generally accepted that there is a plateau of oviduct growth response to estrogen administration related to body size in immature or ovariectomised animals (18). The authors were interested mainly in examining the changes taking place in oviduct tissue growing at a rate comparable to that in the female fowl prior to the onset of laying. Most investigations of the effects of estrogen on oviduct growth in the chick have employed low levels of hormone administration which do not produce oviduct growth comparable to that occurring in the pullet (3). Preliminary experiments had shown that female chicks of 300–400 gm. body weight exhibited maximal oviduct development when 10 mg. estradiol was administered over a period of ten days. Dose-related oviduct growth responses therefore were to be expected below this level of hormone treatment.

The levels of hormone used in Experiment 1 (Table 1) gave oviduct

TABLE 1. EFFECT OF ESTRADIOL ON OVIDUCT WEIGHT, MOISTURE CONTENT AND RESPIRATION RATE

(Experiment 1. Mean values)

Total dose estradiol dipropionate (mg.)	Body weight (gm.)	Oviduct weight (gm.)	Dry matter (%)	Respiration rate Q_{O_2}
0.005	350	0.071	13.81	2.33
0.04	345	0.078	14.56	2.79
0.20	359	0.200	15.88	2.44
0.50	326	0.363	16.28	2.47
1.00	342	1.590	16.88	3.51
5.00	353	4.684	18.16	2.51

weights 1.5 to 100 times as great as those in untreated controls where the weight was known to be 50–75 mg. at this age (3, 19). The data for the moisture content of the tissues support those obtained by Brown and Jackson (4), who showed that the smaller oviducts of folic acid deficient chicks treated with estrogen contained a higher percentage of moisture. The respiration rates of the tissues in Experiment 1 showed some stimulation of respiration at the 1.0 mg. level, but at the highest level of 5.0 mg., where there was a large increase in tissue weight the respiration rate per unit dry weight fell.

These data provide preliminary evidence of the rate of oxygen consumption of chick oviduct tissue over a range of growth responses from increasing estrogen doses. In Experiments 2 and 3, three dosage levels of estradiol were selected for further investigation, so that one level was on the plateau of oviduct response and the other two levels in the region of oviduct growth response to estradiol dependent on dose. The results of these experiments are given in Table 2. Good agreement was obtained between the two experiments in relation to the magnitude of the response obtained from the

TABLE 2. THE EFFECT OF ESTRADIOL ON THE WEIGHT, MOISTURE, PROTEIN, FAT AND RESPIRATION RATE OF THE OVIDUCT

(Experiments 2 and 3. Mean values ±S.E.M.)

Total dose estradiol dipropionate	(mg.)	0.75	1.5	10.0
Number of chicks.	Exp. 2	7	7	7
	Exp. 3	9	9	9
Final body weight (gm.)	Exp. 2	356	358	367
	Exp. 3	353	330	345
Fresh weight of oviduct (gm.)	Exp. 2	1.05 ±0.08	1.74 ±0.24	6.24 ±0.33
	Exp. 3	0.70 ±0.11	1.79 ±0.21	6.46 ±0.25
Dry matter (%)	Exp. 2	15.71 ±0.31	15.31 ±0.27	18.83 ±0.72
	Exp. 3	15.83 ±0.62	15.09 ±0.34	19.14 ±0.33
Crude protein % fresh	Exp. 2	12.66 ±0.43	11.77 ±0.47	14.50 ±0.68
Crude protein % dry	Exp. 2	80.70 ±0.65	76.84 ±1.90	77.01 ±1.59
Fat % fresh	Exp. 2	1.72 ±0.08	1.77 ±0.05	2.58 ±0.17
Fat % dry	Exp. 2	10.94 ±0.42	11.56 ±0.26	13.68 ±0.80
Respiration rate (Qo_2, µl./mg. dry wt./hr.)	Exp. 2	3.91 ±0.36	4.24 ±0.36	2.62 ±0.16
	Exp. 3	3.84 ±0.32	4.10 ±0.29	2.16 ±0.10

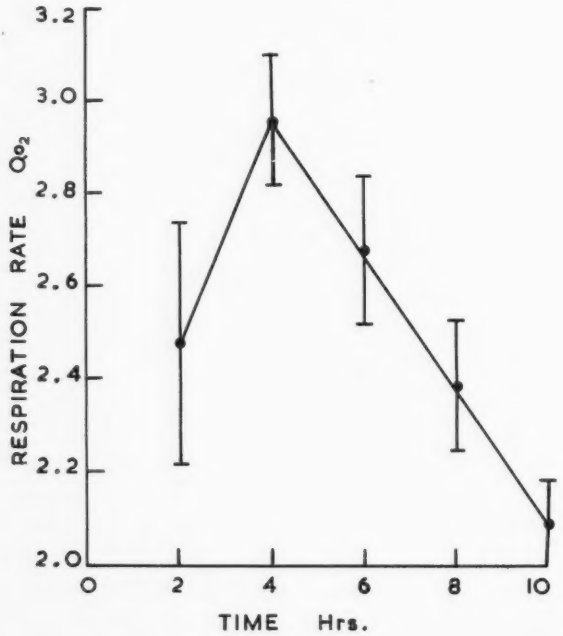

FIG. 1. Effect of time after estrogen injection on oxygen consumption of oviduct tissue. Each point represents mean i.e. Qo_2 (µl./mg. dry wt./hr.) ±S.E.M.

hormone treatments. There was a significant difference between the 0.75 mg. and 1.5 mg. levels of estradiol treatment (Exp. 2, $P < 0.02$; Exp. 3, $P < 0.001$) and between 1.5 mg. and 10.0 mg. levels (Exps. 2 and 3, $P < 0.001$) as regards the fresh weight of the oviduct. The moisture content of the oviducts from birds receiving 10 mg. estradiol was significantly lower than that of the oviducts from birds receiving 1.5 mg. and 0.75 mg. estradiol (Exps. 2 and 3, $P < 0.001$). There was a significant increase in the percentage of protein in fresh oviduct tissue from birds receiving 10 mg. estradiol ($P < 0.01$) as compared with the two lower treatments. This was due to the large influence of moisture content differences on composition. An increase in the fat content of the oviduct associated with a higher level of hormone treatment was observed and this increase attained significance at the highest level of hormone treatment in both fresh ($P < 0.001$) and dried tissues ($P < 0.01$).

The respiration rates of oviduct tissues obtained from chicks in Experiments 2 and 3 are also presented in Table 2. A total hormone dose of 1.5 mg. estradiol gave the highest respiration rate recorded in both experiments. This respiration rate was almost twice that of the oviducts from birds receiving 10 mg. estradiol (Exp. 2, $P < 0.01$; Exp. 3, $P < 0.001$). The lower oxygen consumption of the most highly stimulated oviducts may not be inconsistent with the increase in respiration associated with estrogen administration reported in uterine tissues from the rat and mouse where active growth stimulation is taking place (20). Oviducts from birds receiving 0.75 and 1.5 mg. estradiol were in a state of active development while those from birds receiving 10 mg. had reached the plateau of end organ growth response.

Several workers (9, 10) using small doses of estrogens have investigated the effect of the time lapse after hormone treatment on the respiration rate of rat uterine tissue. Roberts and Szego (10) found that total oxidative metabolism of the tissue reached a maximum 16–20 hours after estrogen treatment. Some indication of an increase in respiration was noted, however, in the first hour. The authors have studied this aspect using oviducts stimulated to the growth plateau since this eliminated possible interference from tissue growth. The respiration rates at increasing times after a test dose of the hormone (2 mg.) are shown in Figure 1.

The respiration rates were of the same order as those recorded for the highest hormone treatments in Experiments 2 and 3.

While there was an indication that at 4 hours after estrogen injection there was an increase in oxygen consumption, significant decreases from this figure were recorded between 4 hours and 8 hours ($P < 0.05$) and again between 6 hours and 10 hours ($P < 0.01$). This decrease occurred earlier than that reported for rat uterine tissue.

It seems well established that one of the best indices of growth pattern is provided by examining the increases in the nucleic acid content (particu-

TABLE 3. EFFECT OF ESTRADIOL ON THE DNA AND PNA CONTENTS AND ON THE ALKALINE PHOSPHATASE ACTIVITY OF THE OVIDUCT

(Experiments 2 and 3. Mean values ±S.E.M.)

Total dose estradiol dipropionate (mg.)	0.75	1.5	10.0
Experiment 2			
Number of chicks	7	7	7
DNA (mg./gm. fresh)	5.72 ±0.16	5.38 ±0.23	4.82 ±0.24
DNA (mg./gm. dry)	36.50 ±0.99	35.15 ±1.42	26.61 ±1.59
Total DNA per oviduct (mg.)	6.01	7.58	29.60
PNA (mg./gm. fresh)	7.25 ±0.37	6.60 ±0.59	8.14 ±0.17
PNA (mg./gm. dry)	46.17 ±2.27	43.10 ±3.92	43.25 ±1.34
Total PNA per oviduct (mg.)	7.66	9.71	50.23
PNA/DNA	1.26 ±0.37	1.24 ±0.13	1.72 ±0.08
Experiment 3			
Number of chicks	9	9	9
Alkaline phosphatase (K.A. units/gm. wet)	0.59 ±0.04	0.74 ±0.10	0.54 ±0.14
Alkaline phosphatase (K.A. units/gm. dry)	3.74 ±0.22	4.84 ±0.64	2.38 ±0.70

larly PNA) of rapidly growing tissues (20). These increases in nucleic acid content are usually accompanied by a parallel increase in alkaline phosphatase activity (21). The authors have determined nucleic acid and alkaline phosphatase levels in oviduct tissues from the experimental birds (Table 3). The level of DNA in dried oviduct tissues from the birds receiving the highest level of hormone was significantly lower ($P < 0.01$) than that in the two lower treatments. This difference fails to attain statistical significance when calculated on a fresh tissue basis. The very great increase in total DNA content of an oviduct indicates, however, that the usual hyperplasia associated with estrogen treatment has taken place. The ratio of PNA to DNA in the oviducts from the birds which received 10 mg. estradiol was significantly greater than in those from birds which received 1.5 mg. estradiol ($P < 0.01$) and gave an indication of a relatively larger increase of PNA and DNA due to estrogen stimulation. The fact that the PNA/DNA ratio was unchanged between the two lower hormone treatments, even though there was a large increase in fresh weight, suggested that this increase in fresh weight was due to hyperplasia, whilst the highest level of hormone produced hypertrophy as well.

Davis, Meyer and McShan (7) have reported that estrogen decreases the alkaline phosphatase concentration per unit fresh weight in rat uterine

TABLE 4. EFFECT OF ESTRADIOL ON THE DRY MATTER AND FAT CONTENT OF BLOOD SERUM

(Experiments 2 and 3. Mean values ±S.E.M.)

Total dose estradiol dipropionate (mg.)	0.75	1.50	10.0
Experiment 3			
Number of chicks	9	9	9
% dry matter	5.72 ±0.20	6.20 ±0.30	20.63 ±0.61
Fat (% fresh weight)	0.55 ±0.05	1.13 ±0.20	12.67 ±0.60

tissue. We have investigated alkaline phosphatase levels of estrogen stimulated oviducts (Table 3). The changes in alkaline phosphatase activity were similar to those for respiration rate. When maximum hypertrophy had taken place the enzyme activity per unit weight decreased ($P < 0.02$). The concentration of this enzyme found in the tissue reflected the degree of cell hypertrophy, an effect also noted by Davis *et al.* (7) with uterine tissue from aminopterin-treated rats. Brown and Jackson (4) have shown alkaline phosphatase activity in the oviduct to be greater in estrogen-treated folic acid deficient birds, which had a decreased growth response to estrogen, than in estrogen treated normal chicks. In the present experiments where the size of the organ was determined by estrogen level rather than folic acid deficiency, the same pattern existed, suggesting that this phenomenon was unrelated to effects other than the degree of hypertrophy of the tissue. Histological observations by Andrus and Zarrow (22) showed that alkaline phosphatase activity in the epithelial layers of the chick oviduct was greatly increased in larger oviducts. In the sampling of whole tissues, as in the present experiment, this effect would be obscured.

The data on dry matter and fat contents of the blood sera from the estrogen-treated chicks indicated the magnitude of the influence of the different levels of hormone on blood composition (Table 4). The highest level of estrogen treatment, which gave maximal oviduct response, also produced marked lipaemia, but there was also an increase in fat content of serum associated with the administration of 1.5 mg. of hormone. Changes in the dry matter of blood serum followed a similar pattern.

Acknowledgments

The authors thank Professor J. Houston for his interest and advice and Mr. G. L. Chambers for technical assistance. One of us (H.G.B.) is indebted to the British Egg Marketing Board for a Research Studentship.

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THE RELATIVE THYMOLYTIC ACTIVITIES OF CORTICOIDS USING THE OVARIECTOMIZED-ADRENALECTOMIZED MOUSE[1]

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ABSTRACT

The thymus gland of the adrenalectomized-ovariectomized mouse is a suitable test object for the bioassay of corticoids administered subcutaneously or orally. The response consisted of an inverse relationship between dose and thymus size. When a total of 44 to 56 mice were used on the standards plus unknowns (two concentrations of each), an index of precision (λ) of 0.221 (range 0.118 to 0.408) was found for the assay of cortisone and cortisol. Increasing doses of cortisol and cortisone and some related steroids produced increasing thymic involutions to 25% of the control size. Desoxycorticosterone produced statistically significant involutions, but the curve appears to level off at 75% of the initial thymus size. On this basis, desoxycorticosterone appears to give a qualitatively different response than that found for the cortisol and cortisone group of steroids. No true thymolytic effect could be demonstrated for the estrogens estradiol-17β and stilbestrol, but testosterone and progesterone produced significant but limited thymus involution at relatively high dosage levels.

When the activity of cortisol, by the subcutaneous route, was assigned an activity of 100%, the following relative activities were found: 9α-fluorocortisol, 272% $\pm$11; cortisone, 79% $\pm$4.0; 9α-hydroxycortisol, 57% $\pm$5.1; corticosterone, 45% $\pm$4.5; 21-desoxycortisol, 35% $\pm$2.5; and desoxycorticosterone, 12% $\pm$0.6.

UNDER the influence of corticoids the thymus gland undergoes involution, and this response has been used for the evaluation of the relative potencies of these steroids (1–8).

This paper, employing a modification of the method of Santisteban and Dougherty (3), reports the relative activities of various corticoids administered both orally and by subcutaneous injection.

METHODS

Mice of the C-57 brown strain are bilaterally adrenalectomized and ovariectomized at 25–26 days of age under ether anesthesia. In most tests, one day after operation, the active material suspended in Tween 80 was administered by subcutaneous injection. The total dose was contained in 0.2 ml. Two injections of 0.1 ml. each were spaced at least four hours apart. Autopsies were performed 18–20 hours after the last injection,

Received November 17, 1960.

[1] Supported in part by U. S. Public Health Service Grant CY-2193.

TABLE 1. THE THYMOLYTIC ACTIVITY OF CORTICOIDS

Corticoid	Total dosage (μg)	No. of mice	Mean thymus ratio ±S.E.
0	0	669	4.05 ±0.04
Cortisol	63	38	3.48 ±0.11
	125	193	2.99 ±0.06
	250	182	2.24 ±0.06
	500	166	1.65 ±0.05
	1000	10	1.13 ±0.16
Cortisone	63	13	3.07 ±0.31
	125	159	3.13 ±0.07
	250	322	2.62 ±0.05
	500	243	1.79 ±0.07
	1000	26	1.27 ±0.09
Corticosterone	63	7	3.29 ±0.27
	125	17	3.54 ±0.21
	250	18	3.17 ±0.22
	500	21	2.13 ±0.19
	1000	21	1.87 ±0.14
	2000	11	1.39 ±0.08
21-Desoxycortisone	125	25	3.48 ±0.17
	250	37	3.27 ±0.15
	500	36	2.56 ±0.10
	1000	36	2.32 ±0.12
	2000	8	1.71 ±0.19
Desoxycorticosterone	250	9	4.64 ±0.17
	500	54	3.58 ±0.08
	1000	34	3.08 ±0.20
	2000	65	3.00 ±0.10
9α-Hydroxycortisol	34	9	3.35 ±0.24
	63	9	2.59 ±0.37
	125	20	3.09 ±0.25
	250	18	2.84 ±0.21
	500	20	2.45 ±0.23
9α-Fluorocortisol	34	19	3.03 ±0.21
	63	18	2.59 ±0.17
	125	19	1.62 ±0.12
	200	18	1.39 ±0.12

at which time body and thymus weights were determined. The responses were recorded as the ratio of milligrams of thymus per gram of body weight.

In a second series of tests the steroids were incorporated in a high protein diet (9). The dosage, in these studies, was expressed as mg. per kg. of food. The diet containing the test steroids was administered for a two-day period starting with the day of operation, after which time autopsies similar to that previously described were performed.

RESULTS

The results (Table 1 and Fig. 1) illustrate the response of the mouse thymus to subcutaneously administered corticoids using the C-57 brown mouse. This series of compounds represents, mainly, highly active steroids. Parallel dose response curves were found for 9α-fluorocortisol, cortisol, cortisone, corticosterone, and 21-desoxycorticosterone. Desoxycorticosterone showed a distinct effect on the thymus gland, and of interest is the

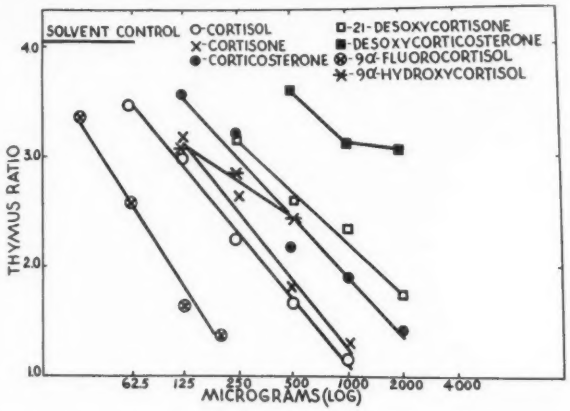

FIG. 1. The relative thymolytic activity of various corticoids.

fact that at least over a narrow dose range the dose-response curve was essentially parallel to that of the more highly active corticoids, such as cortisol and cortisone. 9α-Hydroxycortisol appears to give a unique response insofar as the log dose-response relationship was far more shallow than that observed for the other corticoids. The steroid acetates of cortisol, cortisone, and 11-dehydrocorticosterone, under the same conditions, showed parallel log dose-response curves (Table 2 and Fig. 2).

The influence of the two estrogens, estradiol-17β and stilbestrol, were studied by subcutaneous injection (Table 3). Estradiol-17β, even at concentrations as high as 2 mg. per day, produced no significant involution of the mouse thymus gland. Some irregular non-significant decreases in thymus size were observed at the lower doses of 25 and 250 μg. A similar pattern was found for stilbestrol, where no significant thymolytic effect could be observed at the three concentrations of 0.1, 1 and 4 mg. studied.

Table 4 illustrates the influence of various steroids which are neither

TABLE 2. THE THYMOLYTIC ACTIVITY OF CORTICOID ACETATES

Corticoid acetate	Total dosage (μg)	No. of mice	Mean thymus ratio ±S.E.
0	0	669	4.05 ±0.04
Cortisol Acetate	25	18	4.02 ±0.15
	50	34	2.32 ±0.17
	100	18	1.94 ±0.28
	200	35	1.62 ±0.09
Cortisone Acetate	250	39	2.82 ±0.15
	500	37	2.15 ±0.14
11-Dehydrocorticosterone Acetate	250	18	2.10 ±0.15
	500	28	1.95 ±0.14
	1000	30	1.52 ±0.08
	2000	9	1.16 ±0.13

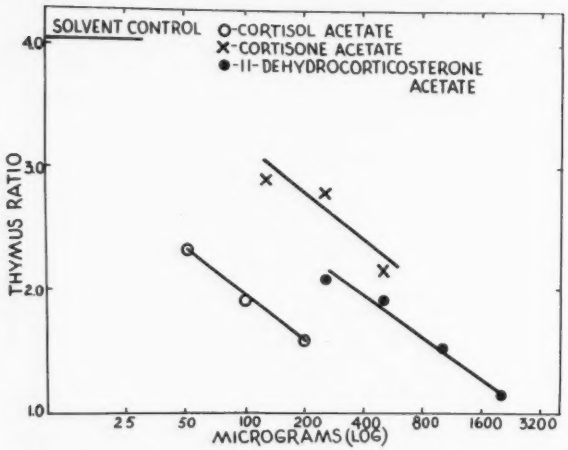

FIG. 2. The relative thymolytic activities of acetates of corticoids.

estrogenic nor true corticoids. Testosterone, at the 1 and 2 mg. levels, produced significant thymolytic effects. However, increasing the dose 4 mg. per day abolished the effect entirely. In the case of progesterone, although three concentrations from 0.5 to 2 mg. were ineffective, the dose level of 4 mg. per day did give a small but significant response.

The thymolytic response to corticoids incorporated in the diet is illustrated in Table 5. Concentrations of cortisol as low as 2.5 and 5 mg. per 100 gm. of food were highly effective in causing thymus involution.

Table 6 illustrates a set of assays employing the involution of the thymus of the adrenalectomized-ovariectomized mouse. Both cortisone and cortisol were assayed against themselves, and the theoretical potency ratio was 100%. In the case of cortisone, when the total number of mice in an assay varied from 44 to 56, potency ratios varying from 66–105% were found. The bioassays were calculated by the method of Bliss (10), in which two

TABLE 3. THE THYMOLYTIC ACTIVITY OF ESTROGENS

Estrogen	Total dose (μg)	No. of mice	Mean thymus ratio ± S.E.
0	0	669	4.05 ± 0.04
Estradiol-17β	5	10	4.26 ± 0.27
	25	18	3.67 ± 0.27
	250	8	3.50 ± 0.31
	1000	19	4.43 ± 0.33
	2000	9	5.08 ± 0.32
Stilbestrol	100	18	3.98 ± 0.38
	500	11	4.01 ± 0.24
	1000	20	3.85 ± 0.22
	2000	11	4.13 ± 0.34
	4000	9	3.73 ± 0.28

TABLE 4. THE THYMOLYTIC ACTIVITY OF MISCELLANEOUS STEROIDS

Steroid	Total dose (μg)	No. of mice	Mean thymus ratio $\pm$S.E.
0	0	669	4.05 ± 0.04
7-Methyl-Δ^5-androstene-3β,17β-diol	1000	8	3.58 ± 0.36
	4000	6	4.38 ± 0.25
^{5}-Androstene-3β,17β-diol	1000	7	3.60 ± 0.23
	4000	7	4.77 ± 0.29
Testosterone	500	8	3.67 ± 0.48
	1000	16	3.11 ± 0.23
	2000	16	2.91 ± 0.30
	4000	24	3.50 ± 0.24
17-Vinyltestosterone	250	16	4.15 ± 0.27
	500	15	4.17 ± 0.34
	1000	17	3.92 ± 0.24
Methyltestosterone	250	7	3.45 ± 0.26
	500	8	3.86 ± 0.32
	1000	7	3.95 ± 0.35
19-Nortestosterone	500	7	3.65 ± 0.31
	1000	8	3.58 ± 0.38
	2000	8	4.20 ± 0.52
Δ^{16}-Dehydroprogesterone	500	8	3.97 ± 0.31
	1000	9	4.03 ± 0.27
	2000	7	3.65 ± 0.38
Progesterone	500	16	4.02 ± 0.24
	1000	35	4.00 ± 0.21
	2000	18	4.20 ± 0.22
	4000	27	3.11 ± 0.28
Δ^5-Androstene-3β,17β-diol	500	9	3.66 ± 0.30
	1000	11	4.43 ± 0.18
	2000	11	3.81 ± 0.31

TABLE 5. THE THYMOLYTIC RESPONSE TO ORALLY ADMINISTERED STEROIDS

Exp.	Steroid	Dose mg./100 gm. food	No. of mice	Mean ratio $\pm$S.E.
A	0	0	11	4.48 ± 0.17
	Cortisol	5.0	9	1.91 ± 0.14
		10.0	10	1.58 ± 0.09
		20.0	11	1.05 ± 0.07
	Corticosterone	5.0	10	3.90 ± 0.25
		10.0	11	1.85 ± 0.18
		20.0	10	1.94 ± 0.23
		40.0	10	1.09 ± 0.09
B	0	0	9	4.57 ± 0.21
	Cortisol	2.5	11	2.47 ± 0.33
		5.0	10	1.44 ± 0.14
		10.0	9	1.11 ± 0.09
		20.0	9	0.94 ± 0.05
	Dehydrocorticosterone	5.0	10	2.59 ± 0.29
		10.0	10	2.36 ± 0.32
		20.0	7	1.57 ± 0.20
		40.0	9	1.31 ± 0.13

TABLE 6. THYMOLYTIC ASSAY OF CORTISONE AND CORTISOL

$$\frac{\text{High Dose of Standard}}{\text{Low Dose of Standard}} = \frac{\text{High Dose of Unknown}}{\text{Low Dose of Unknown}}$$

(Standard = Unknown)

Steroid	Total No. of mice	Ratio of high to low dose (Dose in mg.)	Composite slope b	Significance of slope t	Precision of assay λ	Potency ratio % ± S.E.
Cortisone	56	2 (0.125; 0.25)	−4.54	1.182	0.238	66 ± 13
	44	4 (0.125; 0.5)	−3.35	1.341	0.408	73 ± 21
	52	4 (0.25; 1.0)	−2.48	1.065	0.264	95 ± 16
	44	2 (0.25; 0.5)	−2.42	0.705	0.326	105 ± 22
	52	2 (0.25; 0.5)	−3.45	1.070	0.118	96 ± 12
	52	2 (0.5; 1.0)	−1.69	0.003	0.275	71 ± 14
Cortisol	56	2 (0.25; 0.5)	−1.50	0.954	0.342	107 ± 23

concentrations of the unknown and two concentrations of the standard were employed. In no instance were any significant differences found between the slopes as indicated by the t values listed in Table 6. Lambda (λ), a measure of the precision of the assay, varied from 0.118 to 0.408. In one trial, where cortisol was assayed against itself, a potency ratio of 107 ± 23 was found. Here, the index of precision was 0.342 and the slopes of the unknown and standard were not significantly different.

In another series of assays (Table 7), a three point assay was employed and the data were calculated by the method of Bühlbring and Burn (11). When cortisol was assayed with a theoretical potency ratio of 100%, the

TABLE 7. ASSAY OF CORTISONE AND CORTISOL

(Standard = Unknown)

Standard			Unknown			Combined slope b	Potency ratio % ± S.E.
Steroid	Total No. of mice	Dosage (mg.)	Steroid	Total No. of mice	Dosage (mg.)		
Cortisol	42	0.125; 0.25; 0.5	Cortisol	42	0.125; 0.25; 0.5	−1.50	101 ± 22
Cortisone	42	0.25; 0.5; 1.0	Cortisone	42	0.25; 0.5; 1.0	−2.53	87 ± 9
Cortisone	36	0.125; 0.25; 0.5	Cortisone	35	0.125; 0.25; 0.5	−4.41	80 ± 9

alue found was 101% ±22% and the slopes of the unknown and standard ere not significantly different. Cortisone was subjected to two trials, with otency ratios of 87% ±9% and 80% ±9% being recorded against theo- tical potency ratios of 100%. The relative potency of seven corticoids dministered subcutaneously are listed in Table 8. Cortisol was employed s the reference standard, and its potency was assigned the value of 100%. α-Fluorocortisol was found to be the most active thymolytic steroid, aving a potency ratio of 272% ±11%. The relative potency ratio in de- reasing order was cortisone 79% ±4.0%; 9α-hydroxycortisol, 57% ±5.1%;

TABLE 8. RELATIVE THYMOLYTIC ACTIVITIES OF VARIOUS CORTICOIDS ADMINISTERED SUBCUTANEOUSLY

Standard			Unknown			Combined slopes b	Potency ratio % ±S.E.
Steroid	Total No. of mice	Dosage (mg.)	Steroid	Total No. of mice	Dosage (mg.)		
Cortisol	589	0.063; 0.125; 0.25; 0.5; 1.0	Cortisone	703	0.063; 0.125; 0.25; 0.5; 1.0	−2.12	79± 4.0
			9α-Fluorocortisol	74	0.032; 0.063; 0.125; 0.2	−2.17	272± 11
			Desoxycorticosterone	88	0.5; 1.0	−2.18	12± 0.6
			9α-Hydroxycortisol	58	0.125; 0.25; 0.5	−2.08	57± 5.1
			Corticosterone	88	0.125; 0.25; 0.5; 1.0; 2.0	−2.11	45± 4.5
			21-Desoxycortisone	142	0.125; 0.25; 0.5; 1.0; 2.0	−2.17	35± 2.5

TABLE 9. RELATIVE THYMOLYTIC ACTIVITY OF VARIOUS CORTICOID ACETATES ADMINISTERED SUBCUTANEOUSLY

Standard			Unknown			Combined slope b	Potency ratio % ±S.E.
Steroid	Total No. of mice	Dosage (mg.)	Steroid	Total No. of mice	Dosage (mg.)		
Cortisol Acetate	87	0.025; 0.05; 0.1; 0.2	Cortisone Acetate	76	0.25; 0.5	−1.17	10 ±2
			Dehydrocorticosterone Acetate	85	0.25; 0.5; 1.0; 2.0	−1.02	25 ±6

TABLE 10. RELATIVE THYMOLYTIC ACTIVITY OF VARIOUS CORTICOIDS WHEN ADMINISTERED ORALLY

Standard			Unknown			Combined slope b	Potency ratio % ±S.E.
Steroid	Total No. of mice	Dosages mg./kilo food	Steroid	Total No. of mice	Dosages mg./kilo food		
Cortisol	30	5; 10; 20	Corticosterone	41	5; 10; 20; 40	−4.17	52 ±3
Cortisol	39	2.5; 5.0; 10; 20	11-Dehydrocorticosterone	36	5; 10; 20; 40	−2.26	31 ±4

corticosterone, 45% ±4.5%; 21-desoxycortisone, 35% ±2.5%; and desoxycorticosterone, 12% ±0.6%.

The corticoid acetates were studied and the results are presented in Table 9. Cortisol acetate was found to be ten times as active as the cortisone derivative and four times as active as the acetate of dehydrocorticosterone.

The relative activity of three corticoids, when incorporated in the diet, are listed in Table 10. Corticosterone was found to be 52% ±3% as active as cortisol, and dehydrocorticosterone had an activity of 31% ±4% as compared to the same standard.

DISCUSSION AND CONCLUSIONS

Our experiments indicate that the thymolytic response is not completely specific for so-called corticoids. Rather, it is more correct to say that corticoids of the glycogenic type show a characteristic intense thymolytic effect with a precise relationship between dosage and response. Cortisol, cortisone, and 9α-fluorocortisol are in this class. Desoxycorticosterone produces a small but statistically significant thymus involution. The important point is the fact that desoxycorticosterone produces only a minimum response in spite of rather high doses. It is suggested that although desoxycorticosterone functions as a thymolytic substance, it is qualitatively different in its activity from that of the corticoids of the cortisol group. The difference in activity is not immediately apparent. Desoxycorticosterone is known to have significant and relatively unique inhibitory effects on the oxidative metabolism of various tissues, isolated enzyme system, and an amino acid-protein incorporating system. The question may be asked, "Does desoxycorticosterone produce its limited thymolytic effect by means of this inhibitory action, which is different from the mechanism by which the cortisol group of steroids produce their influence?"

No true thymolytic effects were evident for either the naturally occurring estrogen, estradiol-17β, nor the synthetic estrogen, stilbestrol. Testosterone and progesterone showed some thymolytic activity at high dose levels which may be due to their inhibitory action on oxidative metabolism.

The method as presented here is a practical means to assess corticoid activity. It is simple, fast, and economical. In addition to the usual bioassay precautions, it is important to study not only the ability of a steroid to produce thymic involution, but it is desirable and necessary to demonstrate an extended dose-response relationship. This additional labor is rewarding and would prevent false evaluations of steroids of the desoxycorticosterone type.

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DILATION OF THE UTERINE CERVIX OF THE RAT AND ACCOMPANYING CHANGES DURING THE ESTROUS CYCLE, PREGNANCY AND FOLLOWING TREATMENT WITH ESTRADIOL, PROGESTERONE AND RELAXIN[1]

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ABSTRACT

An *in vitro* method was employed to measure the dilatability and tensile strength of the uterine cervix of the rat during the estrous cycle, gestation and the puerperium. Dilatability, percent water and cervical weight increased throughout gestation to a maximum at parturition. Tensile strength and percent collagen (as measured by hydroxyproline) of the cervix decreased throughout gestation. Within 48 hours post-partum, all measurements tended to return to values seen in the nonpregnant rat. The maximum dilatability, observed at parturition, was found to be due in part to stretching induced by the fetus traversing the birth canal. Relaxin caused similar changes in dilatability, tensile strengh, percent water and collagen in pregnant ovariectomized rats treated with the ovarian steroids. Measurements obtained in non-pregnant, ovariectomized rats treated with estrogen, progesterone and relaxin yielded comparable results but the magnitude of the cervical dilatability was not as great as that observed in the intact, pregnant animal.

BOTH *in vivo* and *in vitro* studies have shown a progressive increase in the dilation of the uterine cervix of the rat during pregnancy (1–5). The initial studies of DeVaal (1) and Uyldert and DeVaal (2) revealed that this phenomenon was not observable in non-pregnant rats, could not be induced with estrogen and was not due to pressure from the distended uterus. Within recent years evidence has been presented to show that relaxin is one of the hormones necessary for dilation of the uterine cervix in the cow (6, 7), sow (8), mouse (9, 10) and rat (5, 11, 12, 13).

The present study was undertaken to determine the respective roles of the ovarian steroids and relaxin on dilation of the uterine cervix of the rat and to compare the results obtained after hormone treatment with that seen in normal pregnancy. The parameters of cervical change used in this

Received November 24, 1960.

[1] These results were presented at the 1st International Congress of Endocrinology, Copenhagen, 1960. Aided in part by a Research Grant (RG-7831) from the Division of General Medical Sciences, Pubile Health Service, NIH.

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study are: 1) dilatability, 2) tensile strength, 3) wet weight, 4) per cent water, and 5) collagen.

MATERIALS AND METHODS

Animals: Handling Procedures

Female rats (150–250 gm. body weight) of the Wistar strain were divided according to age and housed in groups of 8–12 rats per cage in a room with a controlled 13 hour artificial photoperiod (7:30 A.M. to 8:30 P.M.) and a temperature range of 68°–72° F. Animals were fed a diet consisting of either Rockland Laboratory Feed or Purina Rat Chow in pellet form, and tap water *ad libitum*. Male rats used for mating were similarly housed.

Ovariectomized animals were rested at least two weeks post-operatively before treatment with the ovarian steroids (estradiol 17-β and progesterone in peanut oil) and relaxin[3] (in beeswax-oil) was initiated. Pregnant rats were ovariectomized on day 12 of gestation and injected twice daily with various doses of estradiol and progesterone. Relaxin was injected in a beeswax-oil suspension and administered on days 12, 14, 16, and 18 of pregnancy in one set of experiments, or from days 17 through 19 of gestation in a second group of experiments. All pregnant, ovariectomized animals were killed on day 20 of gestation, i.e., 12 to 16 hours after the last injection of the hormones.

Measure of Dilatability and Tensile Strength of the Cervix

Each animal was killed with ether, weighed and the uterine cervix removed with two incisions, one above the connective tissue ring separating the cervix from the uterus and the other just below the junction of the cervix with the vagina. The tissue was quickly trimmed and weighed to the nearest 0.1 mg. on a torsion balance and moistened with a few drops of 0.85% saline solution. Two stainless steel S-hooks were inserted through a single cervical canal and the tissue was placed on the apparatus depicted in Figure 1.

With the after-load screw (A) of the apparatus (Fig. 1) set to impede lever movement the cervix was inserted by means of pin-hooks onto the muscle clamp and lever arm (B and B′). Slack was taken up by the adjusting clamp (C). The timer (D) was started 5 seconds before "time zero" and during this time the weight pan (E) was lifted off the hook (E′). At "time zero" the afterload screw (A) was released and 45 seconds later the "zero load" reading was obtained from the rule (F). At this time the weight pan (E) which weighed 18 gm. was placed on the hook (E′) and at one minute and 45 seconds, the reading with the pan included in the system was recorded. During the next 15 second interval the pan was removed, the value for the unloaded system was recorded, the pan replaced, and a 10 gm. lead weight was added to the system. Throughout this period the tissue was moistened with saline. The procedure was continued until the cervix broke or until a minimum of 900 gm. had been added, depending on whichever occurred first.

The length of travel of the lever arm was at all times limited from 3.0 cm. above the horizontal to 3.0 cm. below the horizontal. In this manner, the apparent shift of the weight pan toward the fulcrum (G) as the lever traversed an approximate 15° arc did not produce an error in dilatability or stretch of more than 2–4%.

[3] The relaxin (Releasin powder No. W1164A, Lot 66) and the beeswax-oil mixture (5% beeswax in peanut oil, Respository Injection Placebo No. W1164-9P, Lot 006) were kindly supplied by Dr. Robert L. Kroc, Warner-Chilcott Research Laboratories, Morris Plains, N. J. The preparation was assayed against the reference standard (Warner-Chilcott) by Dr. Kroc and was found to contain 150 GPU per mg. of powder.

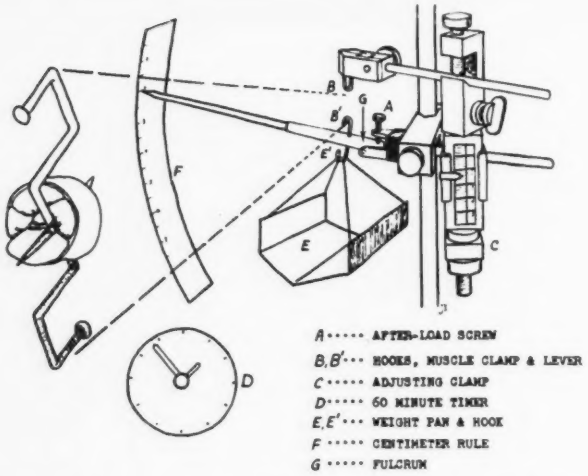

FIG. 1. Apparatus for measurement of dilation and tensile strength of the uterine cervix.

If it became necessary to reset the lever to a new "zero-point" during an experiment, it was done during the 15 second interval, after recording "loaded" and "unloaded" readings. This was done simply by raising hook "B" by means of adjusting clamp "C." A new "zero-point" reading (loaded and unloaded values) was then obtained before placing the next 10 gm. weight on the pan.

The unit weight from which cervical dilatability was calculated was chosen arbitrarily using the following criteria: (a) Measurement of dilation should not include readings obtained at "time zero" as these readings are in some instances inaccurate due to the rapidity of travel of the lever arm, and (b) since the upper limit of the measurement must be obtained before the tissue ruptures, it should be set low enough to include tissues which have a low tensile strength. Using these criteria, it was decided after preliminary experiments that the measure of dilation should be made between the "zero-point" reading of the lever and the reading obtained after the 18 gm. pan and four 10 gm. weights have been added to the system. Hence, cervical dilation was determined from values recorded within the first six minutes of each experiment, after 58 gm. had been added to the system. Since the lever ratio of the apparatus was 10:1 dilatability was calculated as follows:

$$\text{mm. dilatability per 100 gm. weight} = \frac{\text{cm. stretch} \times 100}{58}$$

Tensile strength was calculated as the weight (gm.) necessary to break 1.0 mg. of cervical tissue in a 100 gm. rat (gm./mg. cervix/100 gm. body weight). It should be pointed out that the values for tensile strength as expressed in these experiments are not the true tensile strength of the cervix but an expression of the tenacity of the tissue. Since tensile strength is by definition force per unit area and our values are expressed as force per unit weight, it is obvious that the two are related insofar as the area and wet weight of the cervix are related. Furthermore, since no effort was made to compare different tissues but only to get comparative values, we feel that this type of measurement is valid.

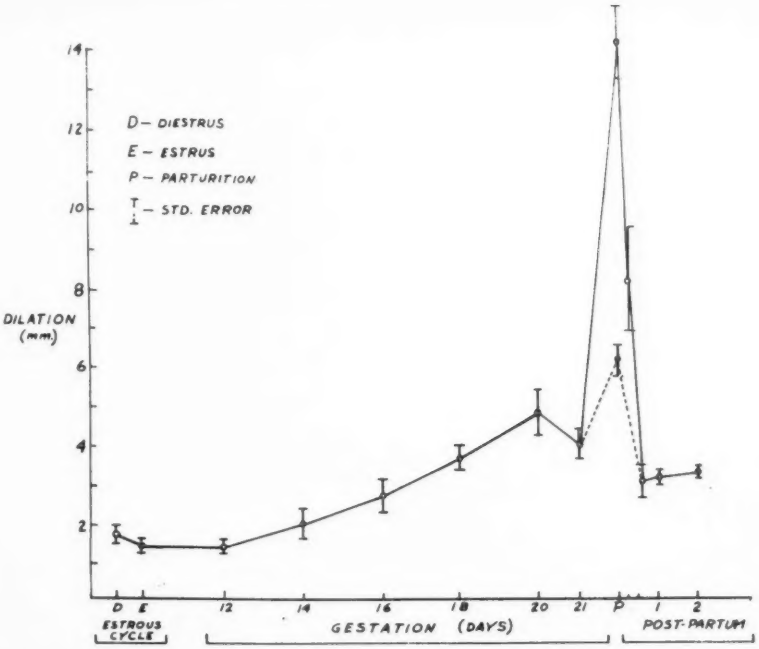

FIG. 2. Dilatability of the uterine cervix of the rat during the estrous cycle, gestation and the early post-partum period. The dotted curve (. . .) represents the dilatability of the cervix of the rat in which delivery had not or was prevented from occurring.

Measure of Collagen

Estimation of collagen of the cervix was performed by measurement of the amount of hydroxyproline of the tissue after drying *in vacuo* at 60° C. The dried cervices were ground in an agate mortar and pestle to a coarse powder. Since the dry weights ranged between 2 and 20 mg., several were usually pooled to make up the required 20 to 50 mg. sample to be analyzed. Each sample was then hydrolyzed in a sealed glass culture tube together with 1.0 ml. of 6 N HCl in an oven for 3 to 5 hours at 138° C according to the method of Levene and Gross (14). The hydrolysate was then filtered and diluted to 25 ml. with distilled water. The estimation of the hydroxyproline content of the hydrolyzed tissues was carried out by the method of Neuman and Logan (15, 16) and read in a Klett colorimeter at 540 mμ. The determination is based upon a color developed by a complex formed between pyrrole-2-carboxylic acid and p-dimethyl-aminobenzaldehyde Per cent collagen in the analyzed tissue was calculated from the following equation:

$$\text{Per cent collagen in tissue} = \frac{100}{13.5} \times \frac{\mu\text{g hydroxyproline in 1.0 ml. final dilution}}{\mu\text{g wet tissue represented in 1.0 ml. final dilution}}$$

RESULTS

Dilation of the uterine cervix during the estrous cycle varied from 1.78 m. during diestrus to 1.45 mm. during estrous (Fig. 2). During pregnancy he dilation increased steadily from a value of 1.44 mm. on day 12 of gesta-

TABLE 1. CHANGES IN THE WEIGHT, WATER CONTENT, AND COLLAGEN OF THE UTERINE CERVIX OF THE RAT DURING THE REPRODUCTIVE CYCLE

Stage of cycle	No. of rats	Cervical weight (mg.)			Water—cervix		Collagen—cervix	
		Wet	(S.E.M.)	Dry	Percent	(S.E.M.)	mg.	Percent
Diestrus	5	31.2 ±	5.23	6.4	79.1	.97	3.0	9.8
Estrus	5	41.6 ±	4.74	9.0	78.1	.42	3.9	9.3
Pregnancy								
12 d.	5	29.2 ±	1.37	5.5	81.2	.70	1.7	5.7
14 d.	5	40.3 ±	4.18	7.2	82.2	.44		
16 d.	5	52.1 ±	2.49	9.2	82.4	.30	2.4	3.6
18 d.	5	83.6 ±	11.12	14.5	82.8	.69		
20 d.	5	89.5 ±	14.66	15.5	82.5	.67	3.4	3.8
21 d.	5	85.5 ±	6.64	14.5	82.2	.55		
22 d.	5	114.7 ±	2.30	21.3	81.5	.42		
Parturition								
22 d.	5	142.9 ±	23.60	23.0	82.8	.39	3.9	2.8
Post Partum								
6 hr.	5	111.4 ±	13.40	13.9	83.0	.48		
12 hr.	5	96.0 ±	8.69	18.0	81.1	.70	5.5	6.6
24 hr.	5	63.6 ±	4.22	13.1	79.3	.24		
48 hr.	5	61.1 ±	5.90	12.5	79.4	.31		

tion to 6.20 mm. in the "preparturient" rat (day 22, undelivered). During delivery, the extensibility increased to an average value of 14.18 mm., and within 12 hours post-partum, the value decreased to 3.12 mm. and remained unchanged at 48 hours post-partum.

Cervical weight during this period (Table 1) increased from 30–40 mg. during the estrous cycle to about 140 mg. at parturition. Within 48 hours post-partum, the weight of the cervix decreased to 61 mg. The weight increase during pregnancy seemed to be a function of both an increase in dry weight and an increase in per cent water. If dilation of the cervix is calculated in terms of mm. stretch per unit weight of cervix (Fig. 3), the values range from 0.25 mm. to 0.36 mm. under all conditions except during and immediately after delivery (dilation, 0.71 mm. and 0.48 mm., respectively) and during estrus and the early post-partum period (0.16 mm. and 0.19 mm., respectively).

Values for the tensile strength of the cervix measured as grams force per mg. percent wet weight of the tissue, were 78.9 gm. during diestrus and 54.1 gm. during estrus (Fig. 4). Tensile strength decreased during pregnancy from 32.6 gm. on day 12 to 4.1 gm. at parturition. Within 48 hours after delivery, the tensile strength was observed to be about 20 gm. Percent collagen (as measured by the hydroxyproline content of the tissue) was observed to decrease in a fashion similar to that observed for the tensile strength, but the collagen content per cervix was observed to increase throughout pregnancy and during the post-partum period (Fig. 5).

In rats ovariectomized on day 12 of gestation and treated with various

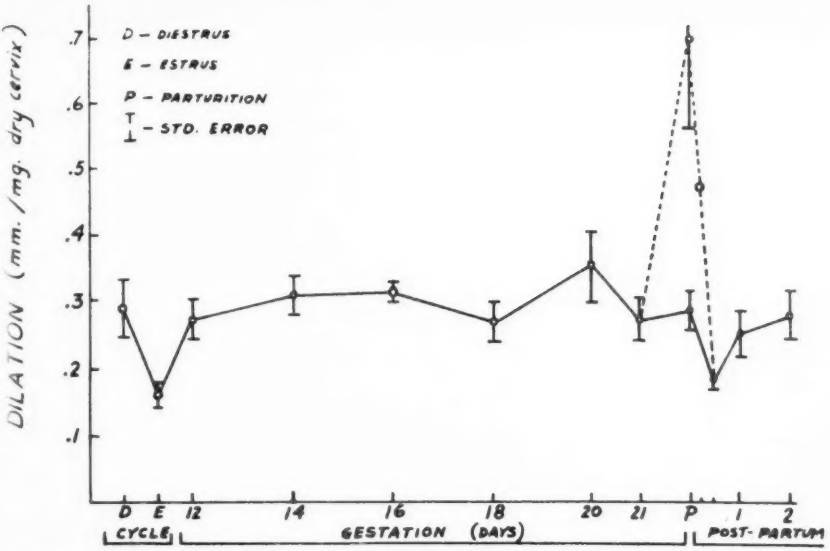

FIG. 3. Dilatability of the uterine cervix per mg. dry weight. The dotted curve (. . .) is based on the dilation obtained in the rat if parturition is allowed to proceed normally.

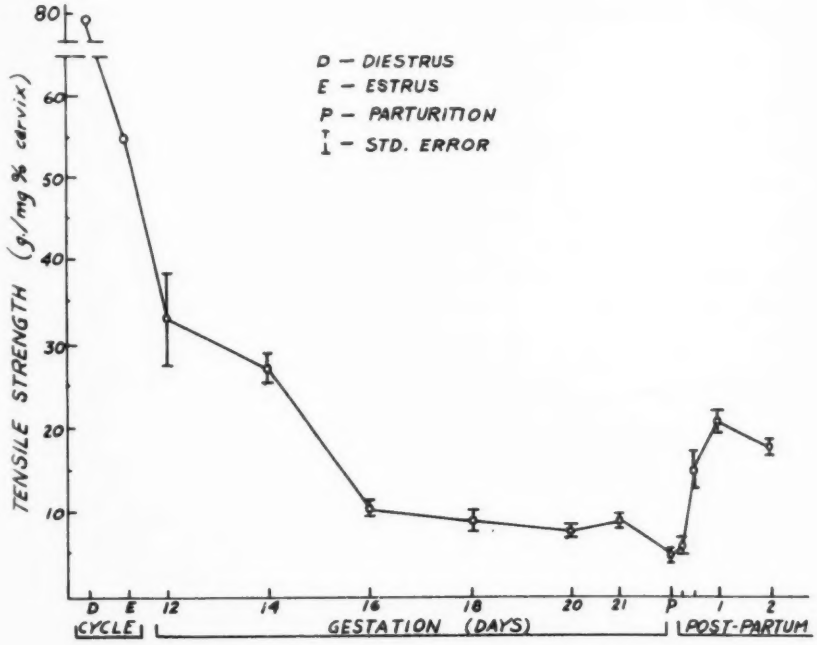

FIG. 4. Tensile strength of the uterine cervix of the rat.

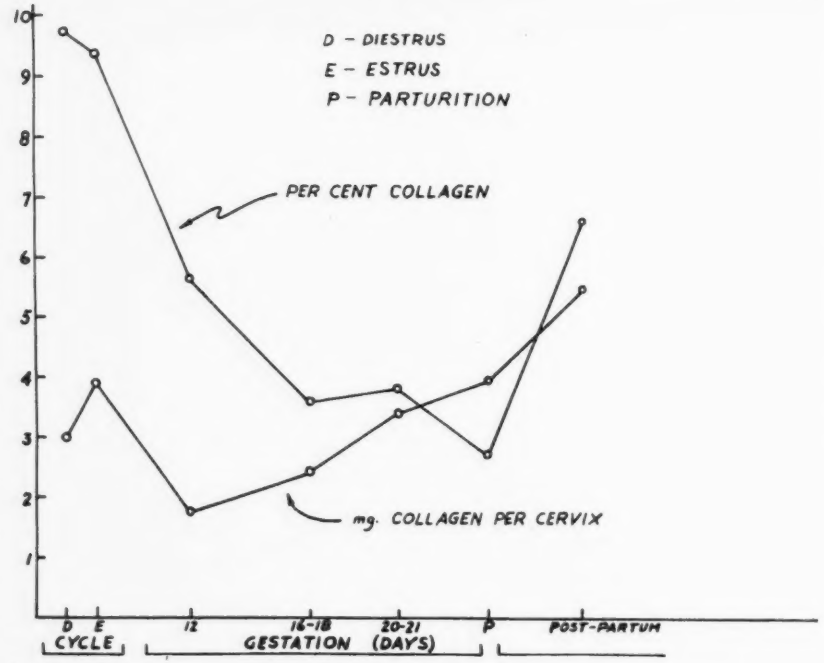

FIG. 5. Collagen content of the uterine cervix of the rat.

combinations of the ovarian hormones, treatment with progesterone alone resulted in cervical dilation of 1.82 mm. (Fig. 6). The survival of fetuses in these rats was 72%. Weight and water content of the cervix were 37.2 mg. and 80.9%, respectively (Table 2). The addition of estradiol to the progesterone regimen resulted in optimum maintenance of gestation (90% survival or greater) and a slight increase in extensibility (as the estrogen: progestogen ratio was increased), as well as an increase in weight and water content (Table 2, Fig. 6). Concomitant administration of estradiol, progesterone and relaxin resulted in changes in the cervix which approximated those observed just prior to or during delivery in the normal animal. Dilatability was observed to be 7.80 mm. in experimental rats which did not deliver spontaneously, and 12.32 mm. in rats which did prematurely deliver. Cervical weight increased to about 100 mg. and contained about 87% water.

Measures of tensile strength of the cervices of castrated, pregnant rats yielded the following results (Table 2): With progesterone alone the tensile strength was 53.4 gm. The addition of estradiol caused a decrease in tensile strength to 10.8 gm., this value was observed with the highest ratio of estrogen: progestogen used in these experiments, i.e., 1:5000. Concomitant administration of all three hormones resulted in a decrease in tensile

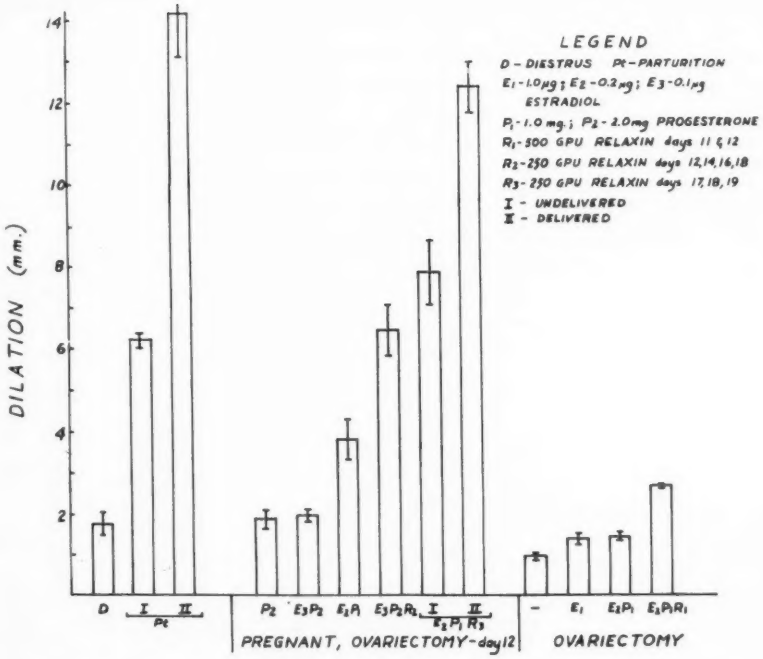

FIG. 6. Effect of estradiol, progesterone and relaxin on cervical dilatability.

TABLE 2. THE EFFECTS OF THE OVARIAN HORMONES ON WEIGHT, WATER, COLLAGEN AND TENSILE STRENGTH OF THE UTERINE CERVIX OF THE RAT

Treatment			No. of rats	Weight (mg.)			Water		Collagen		Tensile strength	
				Wet	(S.E.M.)	Dry	Percent	(S.E.M.)	mg.	Percent	gm.*	(S.E.M.)
E	P	R										
—	2	—	6	37.2	1.68	7.1	80.9	.87	3.2	8.6	54.3	11.5
.1	2	—	5	41.7	1.60	7.9	81.1	.46	3.4	8.1	30.5	5.9
.2	1	—	5	60.4	4.20	10.0	81.9	.54	4.5	7.6	10.8	1.6
—	2	r	5	64.6	6.80	10.7	83.4	.20	3.9	6.0	11.9	1.7
.1	2	r	5	91.7	12.10	12.6	84.3	.52	4.9	5.4	6.1	0.2
.2	1	r′	7	99.9	7.40	13.4	86.9	.71	3.8	3.8	3.6	0.3
Partum			5	142.9	23.60	23.0	83.8	.39	3.9	2.8	4.1	0.7

Rats ovariectomized day 12 of gestation. E—µg Estradiol; P—mg. Progesterone; r—250 GPU Relaxin, days 12, 14, 16, 18; r′—250 GPU Relaxin, days 17, 18, 19; autopsy day 20 of gestation.

E	P	R										
—	—	—	9	8.0	0.78	1.9	76.3	1.13	1.0	12.0	122.5	13.7
1	—	—	5	33.2	1.67	7.2	78.5	.52	2.6	7.9	38.0	2.7
1	—	r	5	46.1	2.45	8.9	80.6	.10	—	—	16.6	0.8
.2	1	—	4	35.0	0.60	7.4	79.3	.50	2.5	7.2	33.2	3.0
.2	1	r′	5	46.2	1.27	7.9	82.9	.24	2.1	4.5	11.3	1.2

Rats ovariectomized. E-1: 1.0 µg Estradiol; r—500 GPU Relaxin days 6 and 8 of treatment; autopsy day 9. E-.2: 0.2 µg Estradiol and 1.0 mg. Progesterone per day for 7 days after priming with 1.0 µg Estradiol for 5 days; r′—500 GPU Relaxin on last two days of treatment.

* gm./mg. cervix/100 gm. body weight.

strength to values similar to those observed during parturition in normal rats. The amount of collagen in the uterine cervix was observed to be between 7 and 9% in pregnant rats treated with only the ovarian steroids. The addition of relaxin to the steroid regimen resulted in a decrease in collagen to 3 to 6% of the wet tissue.

Treatment of ovariectomized rats with estradiol resulted in an increase in weight and water content of the cervix (Table 2) but no appreciable change in dilatability (Fig. 6). Injection of progesterone with estradiol had no effect at the dose levels used. Long term treatment with estradiol cyclopentylpropionate (ECP), or ECP and 2.0 mg. progesterone for 21 days produced results similar to that obtained with the shorter treatment. The addition of relaxin to the steroid regimen resulted in an increase in weight, water content and dilatability but the magnitude of the changes were not as great as those observed in the pregnant rats. Tensile strength and collagen content of the cervices of ovariectomized rats (Table 2) were observed to be about 120 gm. and 12%, respectively. Treatment with estradiol reduced these values to about 40 gm. and 8%, respectively. Progesterone, at the dose levels used had no significant effect, but the addition of relaxin to the combined steroid regimen resulted in values which approximated those observed in normal pregnant rats prior to delivery.

DISCUSSION

Dilatability and Size of the Cervix

The present results on the dilatability of the uterine cervix of the intact rat show a slight drop from diestrus to estrus and a progressive increase in dilation during the course of gestation. This increase culminates in a peak at the time of delivery and returns to preparturient values within 24 hours. These results confirm earlier investigations of a similar nature (4, 9). Recalculation of these data in terms of dilatation per unit weight of dry cervix, however, yield two interesting points:

(1) The increase in dilatability which occurs throughout gestation and in the undelivered, pregnant rat may be in part a function of the size of the cervix, since dilation per unit weight of cervix remains remarkably constant during this period (Fig. 3). Comparison of intact rats during gestation with experimental animals (Fig. 7) shows, however, that relaxin, administered to non-pregnant and pregnant rats caused an increase in both wet weight and dilatability per mg. dry tissue. Thus, the increase in dilation due to the action of relaxin may be a result of both an increase in cervical dimensions as suggested by Cullen and Harkness (12), and a direct effect of relaxin on the cervix, possibly on the integrity of the connective tissue framework. That a rise in dilatability per unit weight cervix as not observed in the intact rat during pregnancy may merely be due to the inadequacy of the measurements or the possibility that the effect due to the increase in the mass of the cervix overshadowed the other effect.

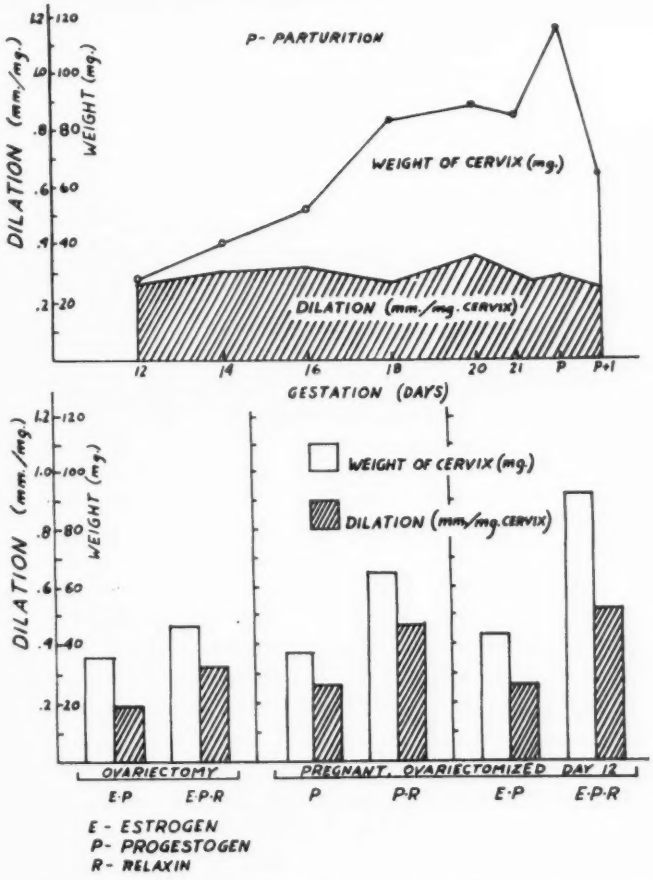

FIG. 7. Dilatability and size of the uterine cervix of the normal pregnant rat, castrated rat treated with estradiol, progesterone and relaxin and the pregnant, castrated rat treated with estradiol, progesterone and relaxin.

(2) A decrease in dilation per mg. cervix, noted during estrus and at 12 hours post-partum, may be a function of the presence of increased amounts of estrogen. (In many mammals, there occurs a characteristic post-partum estrus soon after delivery.) In the ovariectomized rat, it was observed that treatment with estradiol caused an increase in cervical size from about 2 mg. to 7 mg. dry weight, but this increase was not reflected in an increase of similar magnitude in dilatability. Hence, dilation per unit weight cervix decreased from about 0.5 mm. to 0.2 mm. after treatment with estrogen, a phenomenon similar to that observed in the normal rat (17) and sow (18).

The discrepancy between the cervical dilation seen in the castrated pregnant rat treated with estradiol, progesterone and relaxin and the nor-

mal pregnant rat at parturition is explainable on the basis of increased dilatability due to passage of young through the cervical canal. If normal pregnant rats are divided into two groups and dilatability measured on day 22 of pregnancy but prior to delivery in the first group and during delivery in the second group, values of 6 mm. cervical dilation is obtained in the first group and 14 mm. in the second group. Hence it is not too surprising that hormone treatment alone gave a maximum dilation of only 8 mm. However, passage of paraffin rods through the cervical canal of intact pregnant rats prior to delivery increased the dilatability of the cervix from 6 mm. prior to passage to 12 mm., a value comparable to that seen at a normal parturition. It may be presumed, therefore, that the extensibility observed during delivery is in part due to physical effects induced by the fetuses in transit through the cervical canal.

In no case could the degree of extensibility of the cervix observed during the 20th to 22nd day of gestation be reproduced in the ovariectomized, non-pregnant rat treated with any of the combinations of the ovarian hormones. This may be a consequence of the inability of the treatment to achieve maximum cervical size. However, in gonadectomized, pregnant rats treated with the ovarian steroids, the addition of relaxin resulted in an increase in extensibility of the cervix which resembled that observed just prior to delivery. In several instances (Fig. 6) rats treated with the three hormones were observed to deliver prior to autopsy on day 20 of gestation. In these cases, cervical dilation was measured at about 12 mm. This value is comparable to that observed in the normal rat during delivery and tends to substantiate the presumption that the increase in dilatability observed during parturition is due to pressure of the fetuses upon the cervix.

Thus, in the normal course of gestation and parturition in the rat, the increase in extensibility of the uterine cervix seems to be a function of increased cervical growth, increased dilatability per unit weight of tissue and pressure on the cervix during the act of delivery. The action of estrogen was seen in terms of growth of the tissue which apparently resulted in little to no increase in total dilatability, and hence a decrease in dilation per unit weight cervix. Progesterone, at the dose levels used had little to no effect, but relaxin, in combination with both steroids caused the greatest increase in extensibility per unit weight tissue and in growth, both actions resulting in an over-all rise in extensibility of the cervix.

Tensile Strength, Collagen and Water

The present data suggest that a relation may exist between the tensile strength of the cervix and the percent of connective tissue present. These data confirm similar observations by Cullen and Harkness (12). In the intact gestating rat, there was observed a concomitant decrease in both tensile strength and in relative collagen content. Under the influence of estrogen, both in the non-pregnant and protestogen treated pregnant,

gonadectomized rat, tensile strength and percent collagen decreased in a remarkably consistent fashion. Similarly, after treatment with relaxin, both in progestogen and estrogen-progestogen treated animals, a further decrease in tensile strength and collagen content was observed, the greatest changes occurring in the latter group. The fact that the changes in tensile strength parallel closely the changes in collagen and that the hormones which affect one affect the other similarly strongly suggest that the collagen content of the cervix may, in part, be a determining factor of the tensile strength of that tissue.

Measurements of percent water of the uterine cervix of intact rats show an increase from 78% during estrus to about 83% at parturition. These changes were reproduced in pregnant and non-pregnant rats only by combinations of all three of the ovarian hormones. The increase in water content of the rat cervix after injection of relaxin is similar to that reported in the uterus of the rat and the cervix of the sow after similar treatment (18, 19).

From the present series of experiments, it appears that the "softening" of the uterine cervix which occurs after treatment with relaxin is primarily due to a decrease in relative collagen content of that tissue coupled with an increase in water content. These changes might logically result in a decrease in tensile strength. The increase in dilatability observed after treatment with relaxin may be a result of an increase in cervical size (which includes that of the collagen framework) and in addition, an increase in the extensibility per unit weight, the latter perhaps a reflection of the decrease in percent collagen of the tissue. The effects observed after estrogen treatment may result from a differential growth rate which causes an increase in cervical size not matched by an increase of similar magnitude in the dimensions of the collagen framework. Thus, although the percent connective tissue decreases with a concomitant decrease in tensile strength, the connective tissue framework remains unchanged and so dilatability is limited.

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OBSERVATIONS ON THE ENDOGENOUS RESPIRATION OF RAT TESTICULAR TISSUE[1,2]

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ABSTRACT

Changes in the endogenous respiration of testicular tissue following an injury are poorly understood. A nonspecific rise in the Q_{O_2} of injured testicular tissue has been reported in the literature. This study was conducted to determine the possible role of pituitary in the testicular Q_{O_2} changes. Hypophysectomy was found to have no effect on the testicular Q_{O_2}. X-irradiation sharply increased the Q_{O_2}. This increase, however, could be prevented by hypophysectomy. A reversal of the hypophysectomy effect on the Q_{O_2} in x-irradiated testes was produced by gonadotrophin treatment. Also a rise in Q_{O_2} of testes from hypophysectomized animals was produced by gonadotrophin treatment. On the other hand, treatment of intact animals with gonadotrophins did not produce any changes in the testicular Q_{O_2}. Correlations of the various experimental states with the testicular histology and the endogenous respiration of the tissue suggested that the changes in Q_{O_2} are related to the levels of pituitary gonadotrophins.

THE endogenous respiration (Q_{O_2}) of normal rat testicular tissue remains constant after the animal reaches maturity (1). Following an injury to the testes of an adult rat the Q_{O_2} of the testicular tissue rises as shown for the cryptorchid testes by Tepperman *et al.* (2), and nitrofuran treated or x-irradiated testes by Featherstone *et al.* (3). Featherstone *et al.* (3) concluded that the rise of Q_{O_2} is related to absence of the more mature types of the germinal epithelium cells from the damaged testes. Tepperman *et al.* (2) on the other hand, suggested that the disturbed ratio of the germinal epithelium to the Leydig cells is responsible for the changes in Q_{O_2}.

X-irradiated or cryptorchid testes, both having high rate of oxygen

Received November 25, 1960.

[1] A part of this investigation was conducted during the author's service at the Naval Medical Research Institute, Bethesda, Md.

[2] Preliminary report of this work was presented at the 1960 Annual Meeting of the Endocrine Society.

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uptake, are composed essentially of Sertoli and Leydig cells and connective tissue. Since the Leydig cells are under pituitary control it was of interest to investigate the role of pituitary and gonadotrophins in the endogenous respiration of the rat testicular tissue.

MATERIALS AND METHODS

Adult male Sprague Dawley rats (200–240 gm.) were employed throughout the study. The animals received standard laboratory rat food and tap water *ad libitum*. Hypophysectomized rats, purchased from the Hormone Assay Laboratories (Chicago, Ill.), received a supplement consisting of raw horse meat and fresh oranges.

X-irradiation:

Animals, anesthetized with I.P. administered Nembutal, were placed in lead shields which allowed only the scrotum and testes to be exposed to radiation. Following radiation factors were used: KVP—200, MA—25, HVL—0.7 mm. Cu., total filter—0.25 mm. Cu. plus 1 mm. Al, cone size 2.5×2.5 cm., distance—28 cm., R/M—142.1, dose time—4′13″, total "R"—600, technique—local to scrotum with one sheet of backscatter.

Determination of endogenous respiration:

The testes were removed from rats under ether anesthesia and weighed on a Roller-Smith torsion balance. The tunica albuginea and blood vessels were stripped. The testicular tissue was placed on chilled watch glass and quickly divided into several portions. About 120 mg. was weighed with an accuracy of ±0.1 mg. and placed immediately into a chilled Warburg vessel containing 2 ml. of buffer (Krebs-Ringer phosphate, 0.01M, pH 7.4). In the vessels the tubules were gently teased apart with fine needles. A portion of testicular tissue was fixed in Bouin's solution for histological study and one portion was dried in a 100° C oven to constant weight. The Warburg vessels containing testicular tissue were attached to the Warburg apparatus. The water bath was maintained at 37.2° C. The oscillations were set at 110 per minute with amplitude of 2 cm. A 15-minute equilibration period was allowed and then the rate of oxygen consumption (the gas phase being air) was determined at 15 minute intervals for 1 or 2 hours. The Q_{O_2} was expressed as microliters of O_2 per hour per mg. of dry weight of tissue. The technique employed was standard as described by Umbreit *et al.*, "Manometric techniques" 3rd edition (1957). Burgess Publishing Co., Minneapolis.

Gonadotrophin injections:

Two gonadotrophin preparations were employed, Armour pituitary gonadotrophin (FSH), and APL (chorionic gonadotrophin). Animals were injected daily for 20 days with 2 units of FSH and 25 units of APL.

Histological preparation of tissues:

The Bouin's fixed tissues were embedded in paraffin, cut at 5 micra and stained with the PAS—hematoxylin technique.

EXPERIMENTAL

Effect of x-irradiation

Groups of intact animals were sacrificed at time intervals of 20, 30, 45, and 60 days after x-irradiation. At all time intervals Q_{O_2} of testicular tissue from x-irradiated animals was significantly higher than that of the controls. The Q_{O_2} values decreased progressively with time post-irradiation

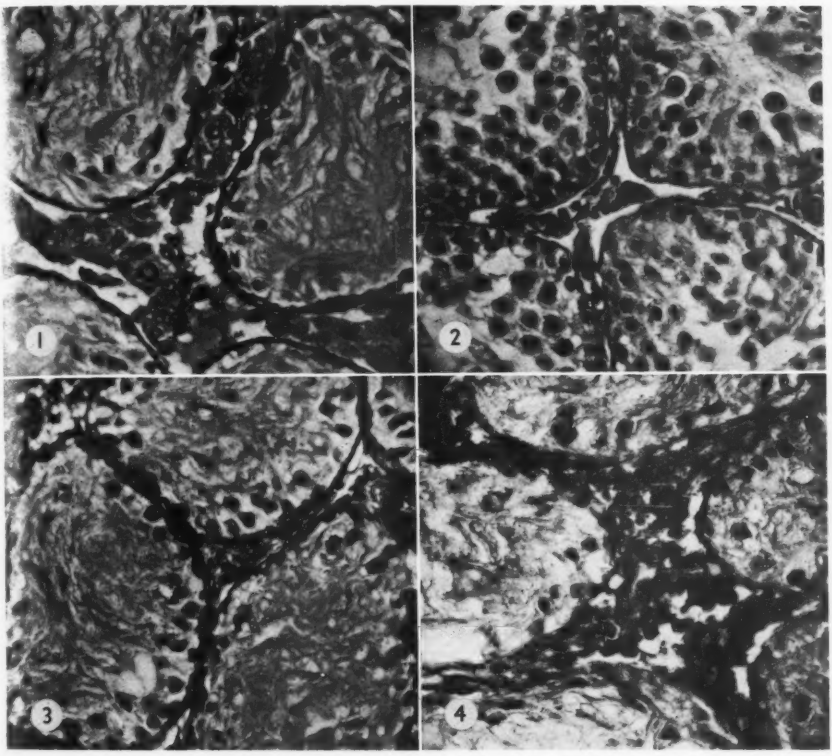

FIG. 1. Testis from a rat sacrificed 30 days after x-irradiation. Note absence of germinal epithelium cells and intact appearing Leydig cells. ×320.

FIG. 2. Testis from a rat sacrificed 33 days after hypophysectomy. Note atrophic Leydig cells and arrest of spermatogenesis at the stage of young spermatids. ×320.

FIG. 3. Testes from a hypophysectomized and x-irradiated rat sacrificed 30 days after x-irradiation. Note absence of the germinal epithelium and Leydig cells. ×320.

FIG. 4. Testes from a hypophysectomized and x-irradiated rat treated with gonadotrophins. Note absence of germinal epithelium and hypertrophy of Leydig cells ×320.

however, all values up to 63 days after irradiation remained well above those of normal controls (Fig. 5).

Histological studies of the testicular tissue revealed at all time intervals complete absence of the germinal epithelium and intact appearing Sertoli and Leydig cells (Fig. 1).

Effect of hypophysectomy

Hypophysectomized rats were x-irradiated 3 days after the operation. Groups of animals were sacrificed at 20, 30, and 43 days after the irradiation; control groups of hypophysectomized but not irradiated animals were

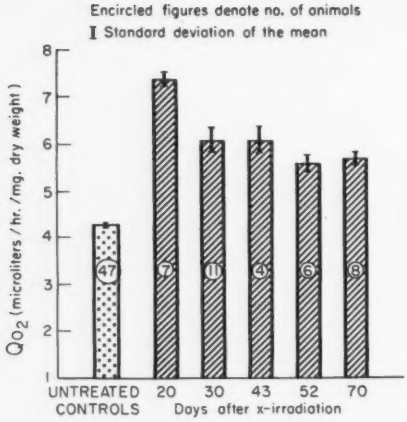

FIG. 5. Effect of x-irradiation on testicular Q_{O_2}.

sacrificed concomitantly (23, 33, and 43 days after hypophysectomy). Results of these studies are summarized in Figure 6. There was no significant difference between the respiration of testicular tissue from intact controls and from hypophysectomized animals. Hypophysectomy, however, completely abolished the increase in Q_{O_2} of testicular tissue from x-irradiated animals. Microscopic study of testes from hypophysectomized animals revealed atrophy of the Leydig cells and spermatogenic arrest at the stage of primary spermatocytes (Fig. 2). The hypophysectomized x-irradiated testes showed, in addition to the absence of germinal epithelium, atrophy of the Leydig cells (Fig. 3).

Effect of gonadotrophins

A group of hypophysectomized animals received 20 daily injections of gonadotrophins (both, FSH and APL) starting on the 23rd day after surgery; the animals were sacrificed following the completion of gonadotrophin

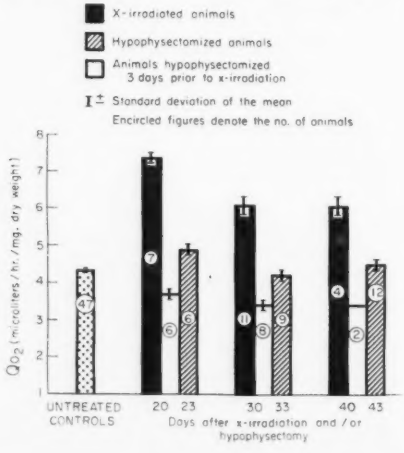
X-irradiated animals
Hypophysectomized animals
Animals hypophysectomized 3 days prior to x-irradiation
I Standard deviation of the mean
Encircled figures denote the no. of animals
Q_{O_2} (microliters / hr. / mg. dry weight)
UNTREATED CONTROLS
20 23
30 33
40 43
Days after x-irradiation and / or hypophysectomy

FIG. 6. Effect of x-irradiation and hypophysectomy on testicular Q_{O_2}.

treatment. A group of hypophysectomized rats was x-irradiated on the 3rd day after the operation and started on 20 daily injections of gonadotrophins 3 days after hypophysectomy. These animals were also sacrificed following completion of the gonadotrophin treatment. A group of intact rats was treated with 20 daily injections of gonadotrophins and sacrificed on day 21. The data from this experiment are summarized in Table 1. Treatment with gonadotrophins did not change the Q_{O_2} of the testicular tissue from intact controls but produced a significant elevation of the endogenous respiration ($P < 0.001$) in hypophysectomized rats. Microscopic study of the testes from both groups of animals revealed good spermatogenesis and numerous Leydig cells. Treatment of hypophysectomized and x-irradiated rats with gonadotrophins raised the Q_{O_2} well above that of normal, hypophysectomized, or hypophysectomized and x-irradiated animals. Histological study of the testes from hypophysectomized x-irradiated and gonadotrophin treated animals revealed reappearance of a large number of Leydig cells while the seminiferous tubules remained devoid of germinal elements containing only Sertoli cells (Fig. 4).

DISCUSSION

A study of the metabolism of testicular tissue poses several serious difficulties to the investigator. First, the testis is an organ composed of two basically different types of tissue, the germinal epithelium, an exocrine organ, and the Leydig cells, an endocrine organ. Each is probably under control of a different trophic hormone. A change in the availability of the respective hormones has a profound effect on the composition as well as metabolic activity of the testicular tissue. Consequently a change in the net metabolic pattern may be the result of changes in metabolic activity of the various cells *per se*, changes in the cellular composition, or both. Results obtained from a study of respiratory activity *in vitro* has to be interpreted in the light of above described relationships as well as in light of the fact that it only provides a rough indication of the algebraic sum of a variety of metabolic activities in the testicular tissue.

TABLE 1. EFFECT OF HYPOPHYSECTOMY AND GONADOTROPHIN TREATMENT ON TESTICULAR Q_{O_2} OF NORMAL AND X-IRRADIATED RATS

	Normal animals	Normal animals - 20 days gonadotropin treatment	Hypophysectomized animals * 20 days gonadotropin treatment	Hypophysectomized animals - sacrificed on the 43rd day	Hypophysectomized x-irradiated animals **	Hypophysectomized x-irradiated and gonadotropin treated animals***
Q_{O_2}	4.3±0.07 #	4.2±0.1	5.5±0.2	4.5±0.13	3.4±0.11	5.2±0.3
	(47)	(10)	(16)	(12)	(10)	(7)
			P 0.001	P 0.001	P 0.01	P 0.001

Parentheses denote number of animals.

Mean ± standard deviation of the mean.

* Gonadotropin treatment started 23 days after hypophysectomy.

** Hypophysectomized 3 days prior to x-irradiation, sacrificed 40 days after x-irradiation.

*** Hypophysectomy and x-irradiation as above, gonadotropin treatment for the last 20 days.

The ability of testicular tissue to utilize *in vitro* added substrates like glucose, pyruvate and oxalacetate appears to be well correlated with the presence of an intact germinal epithelium moiety (2, 3, 4). The changes in endogenous respiration of testicular tissue (oxygen consumption without added substrate) under various physiologic and experimental conditions remain, however, poorly understood. Early studies (1) indicated that the Q_{O_2} of testicular tissue from immature animals is high, drops gradually with age and once the animal attains full sexual maturity it persists at the lower level essentially unchanged throughout the mature life of the animal. This observation was later confirmed by Tepperman *et al.* (2). Damage to the testicular germinal epithelium of a mature animal produces a marked rise of its Q_{O_2} (2, 3). However, according to Schuler (5) the endogenous respiration of testes from hypophysectomized rats (from 2 to 10 weeks after hypophysectomy) is not significantly different from that of normal testicular tissue. Featherstone *et al.* (3) on the other hand indicate that it is increased 10 days after hypophysectomy and remains high up to the 40th day. In our experiments no significant increase was observed for periods from 23 to 43 days after hypophysectomy. No explanation for this discrepancy can be offered at this time. However, the rise in Q_{O_2} of testicular tissue from x-irradiated animals reported by Featherstone *et al.* (3) was confirmed in our studies. An adequate explanation for the rise in endogenous respiration of damaged testicular tissue is not available. Tepperman *et al.* (2) suggested, "In this report the hypothesis is developed that a low oxygen uptake . . . is a characteristic of the germinal component of the testis . . . as the cells of the seminiferous tubules grow . . . their distinctive metabolism obscures the metabolic pattern of the non-germinal elements." Tepperman in this paper suggests that the germinal epithelium has inherent low, while the Leydig cells inherently high Q_{O_2}. Discussing the high Q_{O_2} of cryptorchid rat testes he suggests that in cryptorchid testis with an atrophic germinal epithelium the ratio of germinal epithelium cells to Leydig cells changes markedly in favor of the later and in this fashion "unmasks" the high respiratory activity of the interstitial cells. Although, results reported in our paper, showing that the Q_{O_2} of testis with absent germinal epithelium but essentially intact Leydig cells, is elevated, seem to support Tepperman's hypothesis they also suggest the presence of a different or additional mechanism. It is known that x-irradiation of testes affects the pituitary cytology and gonadotrophin content (6, 7). The results reported here show that the increase in testicular Q_{O_2} following x-irradiation can be prevented by hypophysectomy, while treatment of hypophysectomized and x-irradiated animals with gonadotrophins brings the Q_{O_2} back up. Histological study of the testicular tissue reveals that in all three instances (x-irradiation, x-irradiation plus hypophysectomy, and x-irradiation plus hypophysectomy plus gonadotrophins) the seminiferous tubules are devoid of germinal epithelium; in x-irradiated testes, however, there is abundance of Leydig cells, in hypophysectomized plus x-irradiated testes

the Leydig cells are atrophic and in x-irradiated plus hypophysectomized plus gonadotrophin treated the Leydig cells are present in large numbers. These relationships suggest that the increased Q_{O_2} is related to the presence of the Leydig cells, and the ability of Leydig cells to change their Q_{O_2} is apparently controlled by the gonadotrophins. Since we have used simultaneously both chorionic gonadotrophin and FSH, it can not be stated at this time whether both hormones are essential.

Further evidence showing that "dilution" is not the main factor in the production of Q_{O_2} changes is provided by the following comparison. The increase in Q_{O_2} of cryptorchid testis over normal varies from about 24% to 60% (2), of x-irradiated over normal about 80%. In both instances the testes have no germinal epithelium. The Q_{O_2} of testis in spermatogenic arrest due to nitrofurans is 80% over normal (3). The histological picture of these testes reveals that a large number of germinal epithelium cells is still present in the tubules (8) thus the "dilution" factor should either prevent an increase in Q_{O_2} or at least make the rise considerably less pronounced. This is not the case; actually testes from nitrofuran treated animals show a greater per cent increase of Q_{O_2} than the cryptorchid testes. On the other hand, testes from hypophysectomized rats treated with gonadotrophins showed an increased Q_{O_2} in the face of essentially normal spermatogenesis.

The observation that in x-irradiated testes the originally very high Q_{O_2} changes with the length of time after radiation while the composition of the tubules remains constant (complete absence of germinal epithelium) could be interpreted as providing support for the hypothesis that the changes in the Q_{O_2} are probably under gonadotrophic control. It has been shown by Hildebrand *et al.* (9) that following castration in addition to an absolute increase in the pituitary gonadotrophins there is first an increase in FSH followed by fall in this activity and a rise in ICSH activity. Thus if similar changes occur in the pituitary of rats with testicular atrophy due to x-irradiation the changes in Q_{O_2} could possibly be related to FSH activity. Studies using purified gonadotrophins will be necessary to answer this question.

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THE HYPERGLYCEMIC ACTION OF GLUCAGON AS INFLUENCED BY LIVER GLYCOGEN CONCENTRATION, THE PANCREAS, THE ADRENALS AND THE PITUITARY IN THE BABOON (*PAPIO URSINUS*)

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ABSTRACT

Crystalline glucagon was injected intravenously into a series of 14 intact baboons. The hyperglycemic response was different from one individual to the other but remained similar in the same individual.

The investigation of factors which could be responsible for this inter-individual variation led to the following observations:

1. There was a parallelism between the degree of hyperglycemia and the concentration of liver glycogen only when this concentration was below 1%. At higher concentrations this parallelism no longer existed.
2. The ablation of the pancreas led to an enhancement of the hyperglycemic response to glucagon in the absence of exogenous insulin. When the diabetes was controlled by insulin, the response was not different from that seen in the intact animal.
3. The ablation of the pituitary or of the adrenals led to a decrease in the hyperglycemic response.
4. The concentration of glycogen in the liver of the pancreatectomized, adrenalectomized and hypophysectomized baboons was much above 1%. The effect produced by these endocrine ablations on the hyperglycemic response seemed thus to be independent of the hepatic glycogen concentration.

These observations indicated that the degree of hyperglycemia caused by the injection of glucagon could be dependent not only on the concentration of hepatic glycogen but also on the extent of participation of each endocrine gland in metabolism, which it was suggested varied from animal to animal.

GLUCAGON causes a transient rise in blood glucose concentration. The minimal dose that is required to produce this effect depends on such factors as route of administration, nutritional status and mainly on the sensitivity of the animal species (1, 2, 3, 4).

In a preliminary note (5) dealing with the glucagon sensitivity of the baboon it was reported that the intravenous injection of 15 μg per kg body weight caused an increase in blood glucose of 108 mg.%. When later on the same dose was injected in a greater number of baboons it became

Received November 30, 1960.

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apparent that there was a great variation in response from one individual to the other while in the same individual the response remained similar on repetition of the test on subsequent occasions. Such an inter-individual variation has been reported also in other animal species (6, 7) as well as in man (8, 9). The present work deals with the investigation of factors which may be responsible for the inter-individual variation of the hyperglycemic response observed in the baboons after the administration of glucagon.

MATERIAL AND METHODS

Glucagon Lilly (lot No. 258-234-B-54-2)[2] was injected intravenously into baboons (*Papio ursinus*) at a dose level of 15 μg per kg./body weight. The age of the baboons was between 4 and 17 years and their weight between 11 and 22 kg.

Blood was drawn from the antecubital vein of the unanesthetized animal. One or two samples were taken before the injection of glucagon and one sample 10, 20, 30, 40, 60, 80 and 100 minutes thereafter. Glucose in the blood was determined by the method of Anthanail (10). The glucagon test was performed first in 7 intact baboons 4 hours postprandially. In 4 of these animals the test was repeated several weeks later. The test was further performed in another 7 intact baboons in which in addition a needle biopsy of the liver was taken at the end of the test. The biopsy was analyzed for glycogen by the method of Carroll *et al.* (11). In 4 of these baboons the test and liver biopsy were repeated a second time after 48 hours fast.

Five pancreatectomized, four hypophysectomized and three adrenalectomized baboons were subjected to one or more glucagon tests after complete recovery from surgery. Four tests were done in diabetic animals maintained on insulin and four other tests were done in diabetic animals that had received no insulin for two days. The hypophysectomized baboons received no replacement therapy. In the group of adrenalectomised baboons, one was maintained on DCA alone, a second one on DCA plus 25 mg. hydrocortisone daily, a third on DCA plus 25 mg. cortisone daily prior to one test and on DCA plus 50 mg. cortisone daily prior to a second test.

RESULTS AND DISCUSSION

When glucagon was injected into intact baboons, taken in similar experimental conditions, the degree of hyperglycemia produced, as seen in Table 1, was not identical in all animals, but it remained almost unchanged when on a subsequent occasion the test was repeated in the same individual. It was not possible to establish a correlation between the degree of hyperglycemia on the one side and the initial blood glucose level, the weight of the baboons or their age on the other.

This observation prompted an investigation into the factors responsible for the inter-individual variation in the hyperglycemic response to glucagon.

It is known that glucagon stimulates the reactivation of liver phosphorylase (12, 13) and thus controls the first step in the enzymatic breakdown of glycogen to glucose. It was conceivable that the magnitude of hyperglycemia caused by glucagon was related to the amount of available sub-

[2] Kindly supplied by Dr. O. Behrens of the Eli Lilly Research Laboratories.

TABLE 1. GLUCAGON TOLERANCE TEST IN INTACT BABOONS

Baboon's identification No.	Blood glucose (mg. %)			Time (min.) to reach maximum level
	Initial level	Maximum level	Absolute increase	
347	84	177	93	30
	90	178	88	30
253	94	213	119	30
	105	210	105	30
374	100	186	86	30
394	108	198	90	30
	92	170	78	20
402	121	209	88	30
408	83	120	37	10
	103	154	51	20
306	101	122	21	20

strate. The possibility of such a relationship has been suggested by others (1, 2, 6, 7, 9) but not demonstrated. It thus became necessary to ascertain whether in baboons the extent of the hyperglycemia was related to the hepatic glycogen concentration. Therefore glucagon was injected and the degree of rise in blood glucose was compared with the concentration of hepatic glycogen in each individual baboon. In addition, in order to create greater variations of this last parameter the test was repeated in some of the animals after a 48 hour fast.

It is seen from the results given in Tables 2 and 3 that there is a correlation between the degree of hyperglycemia and the concentration of hepatic glycogen for concentrations below 1% but that for concentrations of 3% or higher this correlation no longer exists. These results indicate that the hyperglycemic response to glucagon is lowered when the amount of glycogen is decreased, thus confirming the suggestion made by others, but these results also indicate that the extent of the hyperglycemic response cannot any more consistently be predicted when the amount of liver glycogen exceeds a certain limit. Obviously over and above the concentration of

TABLE 2. GLUCAGON TOLERANCE TEST AND LIVER GLYCOGEN IN INTACT BABOONS

Baboon's identification No.	Blood glucose mg. %			Liver glycogen %	Time (min.) to reach maximum level
	Initial level	Maximum level	Absolute increase		
308	114	198	84	3.0	30
390	107	187	80	3.2	20
205	100	163	63	3.8	20
365	114	204	90	4.5	20
286	80	103	23	0.3	30
477	93	157	64	4.2	30
488	100	136	36	10.0	20

TABLE 3. GLUCAGON TOLERANCE TEST AND LIVER GLYCOGEN CONCENTRATION IN BABOONS AFTER 48 HOUR FAST

Baboon's identification No.	Blood glucose mg. %			Liver glycogen %	Time (min.) to reach maximum level
	Initial level	Maximum level	Absolute increase		
286	95	115	20	0.05	40
365	108	155	47	0.6	30
205	72	101	29	0.2	40
347	64	70	6	0.01	60

hepatic glycogen other factors are involved in contributing to the inter-individual variation in the hyperlgycemic response to glucagon.

The pancreas, the pituitary and the adrenals are known to play an important role in the regulation of the blood glucose concentration. The possibility was entertained that the rise in blood glucose following the injection of glucagon into the intact baboon was either enhanced or antagonized depending on the extent of participation of each one of these glands in metabolism. The hyperglycemic response to glucagon is altered in diabetes mellitus or after pancreatectomy (2, 6, 14, 15, 16, 17, 18, 19), in Addison's disease or after adrenalectomy (1, 17) after hypophysectomy (20), after treatment with epinephrine (21), with ACTH (7) or with certain steroids (9, 22, 23). In most of the above studies it was assumed but not demonstrated that the altered glucagon response was the indirect consequence of changes in hepatic glycogen concentration resulting from the functional disorders of the endocrine glands or from the administration of hormones. In addition the results reported by different authors were not always consistent. It was thus necessary to re-evaluate the influence of the pancreas, the adrenals and the pituitary on the hyperglycemia caused by glucagon in the baboon and to ascertain whether or not this influence was mediated through changes in liver glycogen concentration.

In order to assess the influence of the pancreas and more specifically that of insulin, glucagon was injected into pancreatectomized baboons either in the presence or in the absence of exogenous insulin. It is evident from the results given in Table 4 that the degree of rise in blood glucose after the injection of glucagon in the pancreatectomized baboon is not dif-

TABLE 4. GLUCAGON TOLERANCE TEST AND LIVER GLYCOGEN CONCENTRATION IN PANCREATECTOMIZED BABOONS MAINTAINED ON INSULIN

Baboon's identification No.	Blood glucose mg. %			Liver glycogen %	Time (min.) to reach maximum level
	Initial level	Maximum level	Absolute increase		
464	122	230	108	5.4	30
475	123	200	73	6.7	20
402	88	195	107	6.0	20
394	78	148	70	—	20

TABLE 5. GLUCAGON TOLERANCE TEST AND LIVER GLYCOGEN IN PANCREATECTOMIZED BABOONS AFTER 2 DAYS OF INSULIN WITHDRAWAL

Baboon's identification No.	Blood glucose mg. %			Liver glycogen %	Time (min.) to reach maximum level
	Initial level	Maximum level	Absolute increase		
481	870	1095	225	3.0	60
402	830	1002	172	—	40
394	606	734	128	2.5	60
	910	1040	130	5.2	60

ferent from that observed in the intact baboon when the diabetes is controlled by insulin. But in the absence of insulin the response is completely different; as seen in Table 5, the blood glucose level continues to rise for a longer period of time and the absolute increase is much greater. Further, Figure 1 shows that the blood glucose level after reaching its peak declines only slowly and remains much above the pre-injection level. It shows also, that the pancreatectomized baboon maintained on insulin is in hypoglycemia at the end of the test. This hypoglycemia is probably independent of the glucagon injection and merely related to the fact that the maintenance dose of insulin by then had reached its maximum effect. In the same animal a decline of blood glucose concentration has been observed in the absence of injected glucagon. Although Figure 1 illustrates the findings of one baboon only, the blood sugar curves behaved in a similar fashion in the other pancreatectomized baboons as well.

Finally it is demonstrated in Tables 4 and 5 that the concentrations of

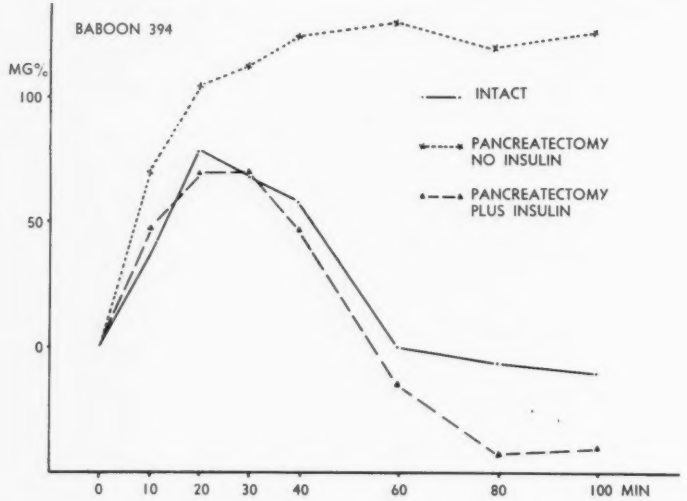

FIG. 1. Increase in blood glucose concentration after the I.V. injection of glucagon, 15 μg/kg. body weight.

TABLE 6. GLUCAGON TOLERANCE TEST AND LIVER GLYCOGEN IN HYPOPHYSECTOMIZED BABOONS

Baboon's identification No.	Blood glucose mg. %			Liver glycogen %	Time (min.) to reach maximum level
	Initial level	Maximum level	Absolute increase		
479	92	112	20	6.4	20
478	85	107	22	8.0	30
413	78	110	32	—	20
434	90	120	30	7.7	10

hepatic glycogen in the pancreatectomized baboons are somewhat higher when they are maintained on insulin. These concentrations are however still in the same range as those observed in the intact baboons.

It is thus possible to conclude that insulin prevents glucagon from producing its maximum effect on blood glucose concentration and this effect is not mediated via changes in liver glycogen concentration.

The hyperglycemic response to glucagon was also examined in hypophysectomized and in adrenalectomized baboons. At the end of each test, except in a few cases where the puncture of the liver was unsuccessful, the concentration of glycogen in the liver was determined. The results are given in Tables 6 and 7 and indicate that in hypophysectomized baboons the hyperglycemic response is low despite the fact that the hepatic glycogen concentration was much higher than in some normal baboons which gave a marked hyperglycemic response. A similar observation was made in the adrenalectomized baboons, except in the one case where the adrenaltomised animal had been pre-treated with large doses of cortisone.

It is evident from these studies, though conducted in a restricted number of animals, that the adrenals and the pituitary are capable of modifying the rise in blood glucose concentration caused by the injection of glucagon, an effect which appears to be independent on the concentration of glycogen in the liver.

Since it seems possible that the glucagon hyperglycemia depends on the functional status of the endocrine glands, the glucagon tolerance test could become an additional way of evaluating in a particular individual the func-

TABLE 7. GLUCAGON TOLERANCE TEST AND LIVER GLYCOGEN IN ADRENALECTOMIZED BABOONS

Baboon's identification No.	Treatment	Blood glucose mg. %			Liver glycogen %	Time (min.) to reach maximum level
		Initial level	Maximum level	Absolute increase		
371	DCA	70	98	28	3.8	30
396	DCA+25 mg. hydrocortisone	65	89	24	—	10
463	DCA+25 mg. cortisone	80	99	19	6.2	10
	DCA+50 mg. cortisone	85	201	116	10.0	30

tional integration of his endocrine system. It has previously been suggested by Gillman and Gilbert (24, 25) that such a system is particular and characteristic for each animal. This particular endocrine configuration in turn could explain at least partially the inter-individual variation in the hyperglycemic response to glucagon on the one hand and the relative intra-individual consistency on the other.

The adrenals and the pituatry have been found to affect liver glucose-6-phosphatase activity (26, 27). It is possible that the low hyperglycemic response observed in our experiments are related to a decrease in activity of this enzyme following the ablation of the pituitary or the adrenals. It is hoped to verify this possibility experimentally.

Acknowledgments

The author wishes to express thanks to Professor J. Gillman for his helpful suggestions and valuable criticism and to Dr. C. Gilbert for making the experimental animals available for this study on glucagon.

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THE EFFECT OF PROLACTIN ON SOME PURINE METABOLIZING ACTIVITIES[1]

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ABSTRACT

Hypophysectomized, oophorectomized rats were injected with prolactin for two weeks. Comparisons with similar rats which received no prolactin, with normal rats and with rats 19–20 days pregnant, showed that prolactin suppressed xanthine oxidizing activity, and that the suppression was more pronounced in mammary tissue than in liver tissue. The loss in xanthine oxidizing activity was accompanied by an accumulation of nitrogen, ribonucleic acid, and deoxyribonucleic acid in the mammary gland. Although injections of prolactin brought the xanthine oxidizing activity to a level which was quite abnormal, they simply maintained the normal levels of adenosine deaminating and guanosine phosphorylating activities, preventing the decrease in these activities which follows hypophysectomy-oophorectomy.

REID, O'Neal and Lewin (1) have reported that hypophysectomy in the rat produces an increase of liver xanthine oxidizing activity. This finding might be interpreted as indicating the presence in the pituitary gland of a factor which suppresses the xanthine oxidizing activity. That this factor is not growth hormone, thyrotropin or adrenocorticotropin is apparent from the experiments of Reid, *et al.* The suggestion that prolactin might be such a factor was made by Chandra, Mendiola and Cole (2) who showed that the injection of prolactin into normal pigeons lowers the xanthine oxidizing activity of liver and especially of crop sac, a tissue whose growth is particularly sensitive to prolactin.

The earlier work was performed on normal pigeons but it is clearly desirable to test the effect of prolactin on xanthine oxidizing activity in animals more comparable to those used by Reid *et al.* (1). The experiments discussed below were therefore carried out using hypophysectomized rats which were also oophorectomized in order to control the endocrinological state of the mammary glands which were to be studied. The effect of prolactin on the xanthine oxidizing activity of the mammary gland (which grows little, if at all, in rats which are hypophysectomized, oophorectomized and injected with prolactin (3)), was compared to the effect of the

Received December 1, 1960.

[1] Supported by a grant (A 2691) from the U. S. Public Health Service, National Institute of Arthritis and Metabolic Diseases, and a grant (G-9870) from the National Science Foundation.

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hormone on the enzymic activity in the liver (which loses weight under such conditions) in order to determine whether the effect on xanthine oxidizing activity is a general secondary effect of tissue growth or is a somewhat more direct effect. The specificity with which prolactin effects xanthine oxidizing activity was tested by comparing the effect on this activity with the effect of the hormone on two other purine metabolizing activities, adenosine deaminating and guanosine phosphorylating activities.

EXPERIMENTAL

Animals. The rats used were females of the Long-Evans strain. Four groups were used: (a) normal rats aged 45–50 days, (b) pregnant rats (19–20 days in their first pregnancy), (c) rats hypophysectomized and oophorectomized at about 26 days of age (and sacrificed at about 40 days), (d) rats which were hypophysectomized and oophorectomized at about 26 days of age, and then injected once daily with prolactin (2 mg. per day) for 14 days. The prolactin used was prepared from sheep pituitary glands by the method of Cole and Li (4), and was estimated to contain less than 0.3% adrenocorticotropin and 2% growth hormone assayed by the method of Lyons *et al.* (5).

The rats were fasted overnight before being sacrificed, and were killed by a blow on the head. The six abdomino-inguinal mammary glands and the liver were removed quickly and placed in ice-cold 0.25 M sucrose solution. After weighing, the tissue was minced with scissors, homogenized in a glass homogenizer fitted with a "Teflon" pestle. The suspension was diluted with cold 0.25 M sucrose to give a 1:10 tissue suspension and in the case of the mammary glad the suspension was strained through cheesecloth to remove connective tissue. Portions of this suspension were used for total nitrogen and nucleic acid determinations.

Chemical Determinations. Total nitrogen was determined by a micro-Kjeldahl procedure. The deoxyribonucleic acid (DNA) content of the mammary tissue suspension was determined by the diphenylamine reaction of Seibert (6), after extracting the nucleic acid from 2 ml. of the tissue suspension as suggested by Schneider (7). The diphenylamine reaction was carried out under the conditions described by Greenbaum and Slater (8). Ribonucleic acid (RNA) was measured by the Albaum and Umbreit (9) modification of the procedure of Mejbaum (10).

Enzyme Assays. Adenosine deaminating activity was measured by the method of Schneider and Hogeboom (11). The procedure followed for the assay of guanosine phosphorylating activity was that of de Lamirande, Allard and Cantero (12). Kalckar's method (13) was used to determine the xanthine oxidizing activity.

RESULTS

An increase in liver xanthine oxidizing activity following hypophysectomy was reported by Reid, *et al.* (1) and confirmed in the present work on hypophysectomized, oophorectomized rats, as shown in Table 1. The increase of the activity in liver by about 50% above normal was accompanied by a doubling of the activity in the mammary gland following hypophysectomy and oophorectomy. When the operated animals are injected with prolactin, however, there is a decrease in the enzymic activity instead of an increase. This suppression of the xanthine oxidizing activity is similar to that found after injecting prolactin into normal pigeons (2). A very striking

TABLE 1. EFFECT OF PROLACTIN ON XANTHINE OXIDIZING ACTIVITY

Type of animal	No. of animals	Mammary tissue				Liver xanthine oxidation‡
		Weight of tissue*	Adenosine† Deamination	Guanosine† phosphorylation	Xanthine oxidation‡	
Normal	12	1.47±0.34	0.34±0.03	16.8±1.2	0.08±0.02	3.1±0.3
Hypophysectomized Oophorectomized	10	1.31±0.24	0.28±0.03	7.2±0.9	0.16±0.02	4.7±0.5
Hypophysectomized Oophorectomized Plus Prolactin§	10	1.24±0.27	0.58±0.06	23.9±2.1	nil	2.4±0.2
Pregnant (19–20 days)	8	4.4 ±0.6	1.49±0.16	16.0±1.4	0.19±0.04	3.7±0.4

* Weight (gm.) of six abdomino-inguinal glands.
† μmoles substrate destroyed per hour per gm. tissue ± standard error.
‡ μmoles uric acid formed per hour per gm. tissue ± standard error.
§ 2 mg. prolactin each day for 14 days.

similarity between the work with pigeons and the present work is the fact that a more dramatic suppression of the activity was observed in the prolactin-sensitive tissues (the crop sac in the pigeon and the mammary gland in the rat) than in the liver.

In order to determine how specific the action of prolactin is for the xanthine oxidizing activity, two other enzymic activities were assayed, adenosine deaminating and guanosine phosphorylating activities. In Table 1 data are presented which show that hypophysectomy and oophorectomy decrease the concentration of adenosine deaminating activity a small amount, and the concentration of guanosine phosphorylating activity more significantly. The injection of prolactin prevents this drop in activities and in the case of adenosine deaminating activity increases the activity above normal, in the direction of the level found in the well developed mammary gland of late pregnancy. However, in terms of the total activity found in the six mammary glands taken from each animal, the effect of injecting prolactin is seen to be little more than the maintenance of the normal total activity of the glands. Thus although adenosine deaminating and guanosine phosphorylating activities are responsive to injections of prolactin, the hormone seems only to maintain the normal amounts of these activities; this is in contrast to the abnormally low levels of xanthine oxidizing activity produced by prolactin injections.

TABLE 2. THE EFFECT OF PROLACTIN ON CONSTITUENTS OF RAT MAMMARY GLAND

Type of rat	Number of rats	Nitrogen*	Ribonucleic acid*	Deoxyribonucleic acid*
Normal	12	210±26	20± 7	40± 7
Hypophysectomized ($\bar{H}$) oophorectomized ($\bar{O}$)	10	130±60	16±15	33±15
$\bar{H}$, $\bar{O}$, injected with prolactin†	10	360±35	90±20	115± 2
Pregnant	8	480±20	150±20	149±16

* Expressed as mg./100 gm. wet tissue ± standard error.
† 2 mg. prolactin each day for 14 days.

The notion that the changes in enzymic activities actually are significant in the metabolism of the mammary gland was tested by measuring the depletion or accumulation of nitrogen, RNA and DNA of the animals in various endocrine states. Table 2 presents the results of these analyses. Hypophysectomy and oophorectomy appear to result in a small loss of nitrogen, RNA and DNA from the mammary gland, and this loss can be prevented by injections of prolactin into the operated animals. Indeed, the prolactin raises the levels of these three constituents above the normal levels in the direction of values found in the mammary gland in late pregnancy. There is, then, a correlation between the suppression of xanthine oxidizing activity and the accumulation of nucleic acids.

DISCUSSION

The xanthine oxidizing activity has been shown to increase following hypophysectomy-oophorectomy and to be suppressed when prolactin was injected into hypophysectomized-oophorectomized rats. These results allow an explanation of the findings of Reid, *et al.* (1), in that the increase of the xanthine oxidizing activity which follows hypophysectomy is seen to be at least partly due to the resulting loss of prolactin. The notion that the suppression is directly due to prolactin itself, rather than to some contaminating factor, is supported by the observation that the relative tissue specificity of the suppression in liver and mammary gland (as well as in the crop sac of the pigeon) is just as would be expected from the usual specificity of the hormone. The directness of the action of the hormone was further supported by finding that two other purine-metabolizing activities, which were shown to be responsive to prolactin, were maintained at normal levels while the xanthine oxidizing activity was uniquely changed to quite abnormal levels in the mammary gland.

While the relative specificity of the suppression of xanthine oxidizing activity for hormone-sensitive tissues might simply represent a general response of any growing tissue, it may be pointed out that the injection of prolactin by itself into hypophysectomized-oophorectomized rats does not cause much growth of the mammary gland (3), and that the suppression of xanthine oxidizing activity in the liver produced by administration of prolactin to hypophysectomized-oophorectomized rats occurs in the face of a weight loss in the liver. Furthermore, it is clear that a similar explanation for the opposite effect (i.e., that an increase of xanthine oxidizing activity is due to the atrophy of tissue) cannot be made, since growth hormone fails to prevent the rise of the enzymic activity which occurs in the liver following hypophysectomy (1) even though it maintains the weight of the liver following the operation (14). Therefore, this suppression of enzymic activity by prolactin appears to be a more direct effect of the hormone than simply a general result of the growth of tissue.

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THE ANDROGENS OF BOVINE SPERMATIC VENOUS BLOOD[1]

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ABSTRACT

The androgen titer of the peripheral plasma of five adult bulls has been found to be of the order of 3.0 μg per 100 ml.; in one animal, treatment with human chorionic gonadotropin (20,000 units per day for 7 days) did not change this value. Spermatic venous plasma of the adult bull (3 animals) was found to range from 7.8 to 17.7 μg per 100 ml.; prior treatment with chorionic gonadotropin (20,000 units daily) for 6 to 7 days raised the spermatic venous plasma androgen from 49.5 to 85.0 μg per 100 ml. Chemical analysis revealed testosterone and androstenedione, in the ratio 7 to 1, to be the major androgenic substances present in the spermatic venous plasma of the gonadotropin-treated animal. 17-Hydroxyprogesterone in quantity equivalent to that of the latter steroid was also isolated from the spermatic venous blood. The significance of the ratio of testosterone to androstenedione in the testis effluent, which is greater than 1 in the case of the adult bull, the dog and the human, and less than 1 in the young bull, the stallion and the rat, is discussed.

THE first investigation of the nature of the testis androgens was carried out in 1940 by Danby (1) on perfused bovine glands. Since that time testosterone and androstenedione have been characterized in the effluent of the dog testis by direct cannulation of the spermatic vein *in vivo* (2, 3), in the stallion by perfusion of the gland (4) and in the human by perfusion (5) and by cannulation of the vein (6). The two precursors of these androgens, progesterone and 17-hydroxyprogesterone (7), have also been detected in the testis perfusate (4).

The present study was undertaken to assess by means of both bioassay and by chemical characterization, the qualitative and quantitative nature of the androgens of bovine testis blood under gonadotropin stimulation. While this work was in progress, the presence of both compounds in the spermatic venous blood of young calves and adult bulls was reported (8).

Received December 5, 1960.

[1] Supported in part by grants, No. C-4004 and No. RG-3721(C5), National Institutes of Health, U. S. Public Health Service.

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TABLE 1. ANDROGEN CONTENT OF BOVINE PLASMA (EXPRESSED AS TESTOSTERONE)

Animal No.	Treatment	Vein	Citrated plasma (ml.)	Androgen content	
				Total (μg)	μg per 100 ml. plasma
HB-1	none	jugular	720	36.0	7.6
HB-2	none	jugular	900	18.0	3.0
35	none	jugular	1200	25.4	3.2
EB-35	none	jugular	1160	24.0	3.1
EB-35	H.C.G.[1] (7 days)	jugular	925	18.0	2.9
HB-1	none	spermatic	475	55.5	17.7
HB-2	none	spermatic	1145	111.0	14.7
HB-33	none	spermatic	1480	78.0	7.8
HB-35	H.C.G.[1] (6 days)	spermatic	2000	1120.0	85.0
36	H.C.G.[1] (6 days)	spermatic	2540	1070.0	63.0
EB-35	H.C.G.[1] (7 days)	spermatic	2390	790.0	49.5

[1] H.C.G. Human chorionic gonadotropin, (Ayerst A.P.L.) 20,000 units per day.

METHODS

Collection of Blood.[3] Normal Holstein bulls, 3 to 4 years old, were immobilized by means of spinal (procaine) and intravenous (pentobarbital) anaesthetics; heparin was administered (500 μg per 1000 lb.) and the internal spermatic vein exposed and cannulated. Blood in 480 ml. volumes was collected in bottles containing 120 ml. of sodium citrate solution. The cells were separated by centrifugation and both plasma and cells were stored in the frozen state and shipped from Ft. Collins to Miami by air. Jugular blood was also drawn and treated in the same fashion.

Treatment of Animals. Certain of the bulls (see Table 1) were treated with human chorionic gonadotropin (Ayerst Laboratories, A.P.L.[4]) by the intramuscular administration of 20,000 international units per day for several consecutive days immediately prior to the collection of jugular and spermatic venous bloods; the duration of treatment in each case is given in Table 1.

Extraction of Steroids. The extraction of androstenedione and testosterone from the plasma was carried out by a modification of a method for the extraction of progesterone from blood (9). Plasma was thawed and treated with one-ninth volume of 5% aqueous sodium hydroxide to give a final concentration of 0.5%. The solution was extracted immediately three times with an equal volume of peroxide-free ether; after standing overnight the extraction was repeated. The procedure was again carried out with methylene dichloride. The combined ether and methylene dichloride extracts were evaporated to dryness and partially purified by a 4-transfer countercurrent distribution

[3] Details of method to be reported elsewhere.

[4] Thanks are due to Dr. A. Stanley Cook of Ayert Laboratories, New York, for a generous supply of A.P.L. used in this investigation.

TABLE 2. RECOVERY OF TESTOSTERONE-4-C^{14} ADDED TO BOVINE PLASMA

Plasma vol. (ml.)	Added (c.p.m.*)	Recovered (c.p.m.)	Recovery (%)
50	780	650	83
50	780	675	86
50	2240	1600	70
50	2240	1700	75

* Samples counted in duplicate in gas-flow window counter at 30% efficiency; a minimum of 1600 c.p.m. accumulated.

between ligroin (upper phase) and 90% methanol, using a minimum of 100 ml. of each solvent phase. The contents of funnels 3 and 4 were combined and evaporated to a small volume under reduced pressure to remove the alcohol; the "neutral polar lipids" were extracted by dilution with water and extraction with methylene dichloride. Recovery experiments utilizing added testosterone-C^{14} showed that 70% or more of added testosterone was recovered by this procedure (Table 2).

After removal of the neutral steroids, the alkaline plasma (pH 12.0 or higher) was adjusted to pH 8.0–8.5 and the ether extractions repeated. The combined ether extracts were evaporated to dryness and set aside from a later study of their estrogen content.

Bioassay. The extracts of the plasma polar lipids were dissolved in 95% ethanol and bioassayed against testosterone (1–8 μg, in ethanol) in the baby-chick comb assay as described by Munson and Sheps (10); unknowns and standard were administered in .05 ml. dose per day for 7 days to each chick. In this assay, as carried out for this study, both androstenedione and testosterone have approximately the same relative androgenic equivalence within the error of the assay (potency of androstenedione: 1.118, relative to testosterone (95% confidence interval: .851 to 1.503), Savard, K. and W. Kessler, in preparation). Accordingly, the androgen content of the plasmas is expressed in terms of testosterone (Table 1) but could also represent combined testosterone and androstenedione. The androgen titers, given in the last column of Table 1, as well as in Table 3, have been corrected for the dilution of the plasma by the citrate solution used in the blood collection procedure, but have not been corrected for the losses in the extraction procedure.

RESULTS AND DISCUSSION

The androgen levels in the peripheral blood of the animals ranged from 2.9 to 7.6 μg per 100 ml. (Table 1). This value must be considered minimal since no correction for the losses in the extraction procedure has been made. The average value of androgen in the peripheral blood of the bull may thus be considered to be approximately 4.0 μg per 100 ml. of plasma. This value may well explain the failure of other workers (1, 6, 11, 12) to detect andro-

TABLE 3. STEROID AND ANDROGEN CONTENT OF SPERMATIC VENOUS PLASMA FROM GONADOTROPIN-TREATED BULLS

	Total (mg.)	μg per 100 ml.
Total androgen (by bioassay, mean)	4.15	104
Testosterone (chemical isolation)	4.29	107
Androstenedione (chemical isolation)	0.58	14.5
17-hydroxyprogesterone (chemical isolation)	0.49	12.2

gen in the peripheral blood of other species including man, since the minimum significant dose in the chick-comb assay is 1.0 μg per chick (Savard and Kessler), thereby requiring approximately 8–15 μg of androgen and a minimum of 300 to 500 ml. of plasma. It is interesting to note that peripheral blood drawn following the administration of chorionic gonadotropin, (bull EB-35) had no greater androgen content than that from animals receiving no treatment. No study of the chemical nature of the androgens of the peripheral blood was carried out because all was used in the bioassays.

Spermatic venous blood drawn from untreated bulls HB-1, HB-2 and HB-33 (in two instances at the time of collection of jugular blood) contained 7.8 to 17.7 μg of testosterone equivalent per 100 ml. of plasma, with an average of 13.4 μg per 100 ml. This value is not at great variance with the androgen titers (1.6 to 5.0 units per 100 ml.) reported for the bull by Danby (1) and those found in the dog (0 to 7 μg of testosterone per 100 ml.) (3, 20) and the human (1 μg of testosterone per 100 ml.) (6). They are, however, not in agreement with the higher values of testosterone reported for the dog and human spermatic venous plasma by other workers (11, 12). In the recent work of Lindner (8) on bull spermatic blood the androgen titers are reported in terms of hourly outputs, and comparison with our data cannot be made.

The pronounced effect of treatment of the animals with gonadotropin prior to collection of blood from the testis may be seen in the remaining 3 animals (Table 1). Here, the androgen titers ranged between approximately 50 to 85 μg per 100 ml. of plasma. This marked effect of systemically administered gonadotropin is in keeping with its anticipated action on the testis; whether it represents stimulation of the existing steroidogenic elements of the testis or merely an increase in their numbers cannot be inferred in this instance of chronic administration of the hormone. These values of androgen content are slightly higher than those observed in dogs pretreated with gonadotropin (20), where 23.0 μg (range 0–160.0) of testosterone per 100 ml. are reported.

Nature of the testicular androgens

In order to determine the chemical nature of the androgens found in the spermatic venous plasma of the bull, the extracts from those animals treated with gonadotropin (lower three in Table 1) were combined. Bioassay revealed a total of 4.15 mg. of androgen (95 percent confidence limits 3.07 to 5.22 mg.), being somewhat more than anticipated from the values obtained on the individual plasmas.

Chromatography of this extract in ligroin/propylene glycol (13) revealed two distinct steroid zones, corresponding to androstenedione and testosterone; these were eluted. The remaining portions of the chromatogram were eluted, combined with the material which had over-run the paper,

and re-chromatographed in the same system. The same two steroid zones were located, eluted and added to the corresponding eluates from the initial chromatogram. The location of these steroid zones was facilitated by the addition of trace amounts (2–4 μg) of testosterone-C^{14} and androstenedione-C^{14} of high specific activity. The reappearance of the two steroids in the second chromatogram was due to their retention at the origin of the first chromatogram by a heavy deposit of highly immobile material. The "non-steroidal" areas of the second chromatogram were eluted and bioassayed. Only a small amount of androgenic activity (36 to 70 μg of androgen) was found in this extract made from the areas outside the clearly delineated zones of testosterone and androstenedione. This androgenic activity was due to residual amounts of both steroids as indicated by the presence of radioactivity, which by calculation corresponded to 40 to 50 μg of testosterone or androstenedione or both.

The zone of androstenedione was re-chromatographed on paper, eluted, chromatographed on silica gel to remove impurities and measured in methanol at 240 mμ.; an actual quantity of 334 μg was determined. This value was adjustable to the amount present in the original extract by means of the factor provided by the amount recovered of radioactive androstenedione tracer added initially; the corrected amount is given in Table 3.

The testosterone area eluted from the appropriate zone of the chromatograms measured 3.85 mg. of steroid, calculated from the absorption in methanol at 240 mμ. This material was acetylated in pyridine and acetic anhydride and chromatographed on paper in ligroin/propylene glycol (4) to provide a fast moving radioactive zone of testosterone acetate and a slow moving, non-radioactive zone of 17-hydroxyprogesterone (13). Both steroids were re-chromatographed on paper, eluted, chromatographed on silica gel and characterized; a) in the case of testosterone acetate by its coincidence with the radioactive testosterone tracer added and by its absorption spectrum in sulfuric acid, and b) in the case of 17-hydroxyprogesterone by its absorption spectrum in sulfuric acid. The amounts of both steroids adjusted (by the same factor of recovery found for testosterone) to the original plasma extract, are given in Table 3. It would seem from these data that the quantities of androstenedione and testosterone (determined chemically), correspond favorably with the mean androgenic titer (determined biologically), thereby implying that both compounds constitute the principal androgenic components of the testis effluent blood.

The isolation of 17-hydroxyprogesterone from spermatic venous blood of the bull confirms the report of its formation (from acetate-C^{14}) in the perfused stallion testis (4) and the levels reported here suggest that the testis is not an inconsiderable source of this compound.

The ratio of 7 to 1 in the concentration of testosterone to that of androstenedione in the testis effluent found in this study, appears to represent a

TABLE 4. RATIOS OF TESTOSTERONE:ANDROSTENEDIONE FROM TESTES OF VARIOUS SPECIES

Species		Method	Treatment	Ratio T:A[1]	Origin of data—references
bovine	adult	sperm. vein cannulation	H.C.G.[2]	7:1	this study
bovine	adult	sperm. vein cannulation	none and H.C.G.	>3:1	8
bovine	calf	sperm. vein cannulation	none and H.C.G.	.03:1	8
equine	adult	perfusion-acetate-1-C^{14}	H.C.G. pretreatment	0.6:1	4
canine	adult	sperm. vein cannulation	H.C.G.	6:1	3
canine	adult	sperm. vein cannulation-acetate-1-C^{14}	none and H.C.G.	3:1	Mason and Samuels, unpublished
rodent—rat	adult	slices-acetate-1-C^{14}	H.C.G.(?)	no testosterone	17
human	adult[3]	perfusion-acetate-1-C^{14}	H.C.G.	2:1	5
human	adult[4]	slices-acetate-1-C^{14}	tumor	1.8:1	18
human	child[5]	slices-acetate-1-C^{14}	tumor	0.5:1	16

[1] Ratio of testosterone:androstenedione.
[2] H.C.G. =human chorionic gonadotropin.
[3] Savard, K., Dorfman, R. I. and Poutasse, E.; unpublished data.
[4] Embryonal carcinoma of the testis in an adult.
[5] Virilizing interstitial-cell tumor of the testis in a 5-year old boy.

characteristic ratio for the adult bull; ratios in excess of 3 to 1 were recently reported by Lindner (8), who also found that the testes of young male calves secrete androstenedione primarily. This ratio is not constant in adults of different species; the few studies reported in the literature and summarized in Table 4 indicate that testosterone is the major component elaborated in the testes of the adult bull, the dog and a single human, and the lesser component in the stallion, the rat, the young bull and in a virilizing interstitial-cell tumor of the testis occurring in a young boy. The exact significance of the ratios of these two androgens of the testis, both as regards species and age, is not apparent from the sparse data available. Their significance may lie at the species-pharmacologic level. Both androstenedione and testosterone have fairly similar androgenic properties in the chick (see above and P. Munson, quoted in (14)), the mouse and the rat (15); by contrast, testosterone is 16–25 times more androgenic than androstenedione in man (16) and 16 times more active than androstenedione in the bull (19). When the relative androgenic activities of the two compounds will be known for other species, the excess of one substance over the other in the testis effluent of different animals may then take on important biological significance. The authors cannot forego the temptation to suggest that in a particular species a shift in testis production from a less active androgen to a more potent one may underlie in part the maturation of the young male at puberty.

Acknowledgment

The authors wish to acknowledge the competent technical aid of Miss Ann Shaw in the early phases of this work.

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EFFECTS OF NIDROXYZONE ON REPRODUCTIVE ORGANS OF MICE[1]

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ABSTRACT

Nidroxyzone was given to mice of both sexes by drug-diet administration. In adult females estrus was inhibited and significant decreases in the weights of sex organs obtained. PMS antagonized these effects. Immature and adolescent females were not affected by nidroxyzone. In male mice of all ages a testicular atrophy developed which was not prevented by PMS. Low to moderate doses were without effect on seminal vesicles. Gonadotropic content of the pituitary gland appeared to be unaltered.

IN 1950 Prior and Ferguson (1) reported that nidroxyzone,[2] a nitrofuran derivative, caused a reversible spermatogenic arrest in the testes of rats without affecting spermatogonia, Sertoli cells or testicular interstitial tissue. This finding was confirmed by Nelson and Steinberger (2). Both groups of workers (1, 3) noted a lack of effect on female sex organs. Ellman *et al.* (4) reported that cysteine could prevent the nidroxyzone-induced spermatogenic arrest in rats. Nitrofurazone, another nitrofuran derivative, was shown by Nissim (5) to cause a degeneration of seminiferous tubules of mice involving all stages of spermatogenesis including spermatogonia but sparing Sertoli cells.

The present communication deals with some effects of nidroxyzone in male and female mice.

METHODS AND MATERIALS

Four strains of mice were used at different times: C_3H, ZBC (A×Z[C_3H] F_1 females ×Z males), ZAF_1 (Z males×ABCA females) and ABCA. The latter strain are mice obtained by mating only the albino young of ABC mice (A×Z F_1 females×A males) for over ten generations. Mice were fed Purina laboratory chow. In all experiments except those dealing with estrus cycles control mice were fed ground control diet ad libitum, unless otherwise noted. Nidroxyzone and cysteine were given by drug-diet administration (DD) ad libitum. Pregnant Mare Serum (PMS) was injected subcutaneously in a volume of 0.1 cc. per mouse.

Mice were sacrified by the intraperitoneal injection of sodium pentobarbital and

Received December 12, 1960.

[1] This work was supported by grants from the University of Minnesota Medical School and from the National Institutes of Health, U.S.P.H.S. (grant A-3052).

[2] Nidroxyzone (Furadroxyl) was generously supplied by Dr. Mary F. Paul of the Norwich Pharmacal Company Laboratories, Norwich, N. Y.

organs carefully dissected free of all surrounding tissue under a dissecting scope, placed on moistened filter paper in a covered petri dish and weighed in a covered weighing bottle to the nearest 0.1 mg. on a Voland analytical balance. Uterus plus vagina were weighed with intrauterine and intravaginal fluid present. Because of the difficulty of dissecting seminal vesicles of adult mice without either losing some fluid or retaining some, if an effort was made to expel it, the seminal vesicles, bladder and prostate were removed together, placed in formalin for about 15 minutes and then the seminal vesicles dissected clean.

To assay the gonadotropic potency of pituitary glands, donor pituitary glands were removed, placed in absolute acetone for three or more days at room temperature, then ground to a fine powder with an agate mortar and pestle, suspended in saline solution to make a concentration of one pituitary gland in 0.3 cc., refrigerated and used within three days. The pituitary homogenate was injected subcutaneously into twenty-one to twenty-two day old female ABCA mice in three doses of 0.1 cc. each given twenty-four hours apart. Control mice were given three daily subcutaneous injections of 0.1 cc. saline. Autopsy was performed approximately twenty-four hours after the last injection and the weight of uterus plus vagina used as a criterion of gonadotropic potency.

In the studies on estrus cycles only those mice with regular cycles prior to therapy were used. The number of cycles occurring after therapy, excluding the first three days, was compared with the number of cycles during the same number of days prior to therapy. Thus mice served as their own controls. Vaginal smears were obtained by saline lavage.

RESULTS

Estrus cycles

Nidroxyzone was found to induce marked decreases in the incidence of estrus in three strains of mice (Table 1). In C_3H mice this occurred without loss of body weight. In both ZBC and ABCA mice some weight was lost but was insufficient to account for the degree of decrease in estrus. Studies in ZBC mice on restricted control diets have shown that a 10% decrease in estrus occurs for each gm. of weight lost (6). In ABCA mice restricted control diets for 23 days caused decreases in estrus of 16 to 29% with body weight losses of 4.4 to 5.7 gm. (daily food intake of 3.0 to 4.5 gm. per mouse, 8 to 10 mice per group) and a decrease in estrus of 81% with an 11 gm. weight loss (2.0 gm. daily food intake, 7 mice). Discontinuance of nidroxyzone administration allowed a return of normal estrus cycling within a few days.

The addition of cysteine appeared to have only a slight effect on nidroxyzone-induced inhibition of estrus. There was evidence of a mild antagonism to higher concentrations of nidroxyzone (0.3%) and of a slight enhancement of smaller doses (0.2%).

Diestrus induced by nidroxyzone was readily overcome by administration of PMS. In fifteen C_3H mice in prolonged diestrus a single injection of one I.U. of PMS induced estrus in all mice. In ABCA mice a single injection of 4 I.U. of PMS caused estrus in 9 of 10 diestrus mice on 0.3% nidroxyzone and in 7 of 7 diestrus mice on 0.3% nidroxyzone plus 1.0% cysteine. Lower doses of PMS were not tried.

TABLE 1. EFFECT OF NIDROXYZONE ON ESTRUS CYCLES OF MICE

Strain of mice	Conc. of drug in diet	No. mice	Days on diet	% decr. estrus cycles	Change in body wt. gm.
C_3H	0.2% nidrox.	5	32	96	0
	0.2% nidrox.	10	45	80[1]	0
ZBC	0.2% nidrox.	10	40	57[2]	−2.1
	0.3% nidrox.	10	23	95	−4.7
ABCA	0.2% nidrox.	9	23	34	−2.4
	0.2% nidrox. +1.0% cysteine	9	23	53	−2.2
	0.2% nidrox. +2.0% cysteine	10	23	60	−3.5
	0.3% nidrox.	10	23	85	−4.9
	0.3% nidrox. +1.0% cysteine	10	23	51	−5.9
	1.0% cysteine	8	23	27	+0.2
	2.0% cysteine	5	23	24	0

[1] Treatment was ineffective the first 10 to 15 days. Decrease in estrus for the last 20 days was 92%.

[2] During the first 20 days estrus was decreased by 47% and weight loss was 4.9 gm. During the last 20 days estrus was decreased by 71% and body weight increased 2.8 gm.

Intact female mice

Since inhibition in estrus induced by a drug but overcome by gonadotropin suggests that the drug may decrease gonadotropic activity, the effect of nidroxyzone on the weight of sex organs was studied. Nidroxyzone did not alter the weights of sex organs in immature or adolescent mice. In twenty-one day old C_3H female mice on diet for ten days the weights of ovaries and of uterus plus vagina were 3.4 ± 0.2 mg. and 24.2 ± 2.0 mg. respectively in 10 controls weighing 11.9 gm., 2.9 ± 0.2 mg. and 20.0 ± 1.2 mg. respectively in 9 mice on 0.1 % nidroxyzone weighing 12.0 gm. and 2.2 ± 0.2 mg. and 11.6 ± 0.4 mg. respectively in 10 control mice autopsied at twenty-one days of age and weighing 7.4 gm. In five to six week old C_3H female mice on diet for two weeks the weights of ovaries and of uterus plus vagina were 3.8 ± 0.3 mg. and 58.5 ± 10.4 mg. respectively in 11 controls weighing 15.6 gm. and 3.5 ± 0.3 mg. and 60.4 ± 7.8 mg. respectively in 10 mice on 0.1% nidroxyzone weighing 15.4 gm.

Results obtained in adult mice are shown in Table 2. It is apparent that nidroxyzone caused marked decreases in the weights of ovaries and of uterus plus vagina in three strains of adult mice. Administration of PMS (ZAF_1 mice) prevented this inhibition and actually increased sex organ weights above that of the controls. Some loss of body weight was noted in treated mice but that this was insufficient to account for the degree of inhibition of sex organs is shown in the last experiment in Table 2 in which untreated mice were given restricted control diets. Mice on 2.5 mg. of control food per day lost 15% of body weight and had ovaries and uterus plus vagina, calculated as mg.% of body weight, decreased by less than 1% and by 16% respectively. ZAF_1 mice on nidroxyzone lost 17% of body weight and their ovaries and uterus plus vagina, calculated as mg.% of

TABLE 2. EFFECT OF NIDROXYZONE IN ADULT FEMALE MICE AUTOPSIED IN DIESTRUS

Strain of mice	Age in months	Weeks on exp.	Treatment	No. mice	Body wt. (gm.) Initial	Body wt. (gm.) Final	Organ weights as mg. % ± S.E. Ovaries	Uter. & vag.	Adrenals	Pituitary
ABCA	3-4	2	Controls	15	28.5	27.5	60.5±3.6	538±25	31.1	9.6
			0.2% nidrox.	13	28.6	27.8	46.8	492	31.8	10.1
			0.3% nidrox.	15	28.2	26.0	35.8±2.2*	348±20*	31.4	9.3
ZBC	3-4	2	Controls	10	22.0	22.8	48.4±6.1	505±33	35.0	9.9
			0.3% nidrox.	10	22.6	19.9	29.5±1.3*	350±28*	33.7	9.9
ZBC	10-11	4	Controls	7	26.1	26.2	47.8±6.7	628±117	29.6±1.4	8.0
			0.3-0.2% nidrox.[a]	9	29.4	24.9	25.1±1.0*	361±23**	22.7±1.7**	9.0
ZAF_1	2	2	Controls	11	21.9	22.6	53.4±2.4	602±18	—	—
			0.3% nidrox.	11	22.4	18.6	29.7±1.2*	328±17*	—	—
			0.3% nidrox. +PMS[b]	11	22.7	17.5	68.4±3.4*	843±32*	—	—
ZAF_1	2	2	ad lib[c]	9	23.0	24.8	46.2±3.7	553±58	—	—
			3.5 gm.	8	23.0	24.0	47.6±3.1	540±25	—	—
			3.0 gm.	9	23.1	21.6	33.9±1.8*	407±57	—	—
			2.5 gm.	7	21.1	17.4	45.9±2.5	464±54	—	—

* P = < .01 compared with controls.
** P = < .05.
[a] Concentration of drug was 0.3 percent for first two weeks and 0.2% for last two weeks.
[b] Four I.U. of PMS injected subcutaneously three times a week.
[c] All mice in this experiment received ground control diet in daily amounts indicated.

body weight, were inhibited by 44 and 46% respectively. If the actual weight of organs in these same animals is used for computation then the inhibition in ovarian weight was 31% in restricted and 57% in treated animals, and the inhibition in uterus plus vagina was 41% in restricted and 60% in treated mice.

Intact male mice

Table 3 summarizes the effects obtained with nidroxyzone in male mice. The first experiment shows that 0.1% nidroxyzone fed for 10 days to twenty-one to twenty-two day old C_3H mice completely prevented the growth of testes but did not affect the growth of seminal vesicles and did not alter weights of adrenal or pituitary glands. In adult C_3H mice a highly significant decrease in the weights of testes, both as actual weight and as mg. percent of body weight, was obtained with 0.1 and 0.2% concentrations. With the higher concentration a decrease was also obtained in the actual weight of seminal vesicles which was just significant as mg. % of body weight. Adrenal glands showed significant decreases in actual weight but not as percent weight. No differences were obtained in pituitary gland weights. In the third experiment of Table 3 various low concentrations (0.01 to 0.1%) of nidroxyzone were given to adult ABCA mice to test for the possible anti-androgenic effect of low doses mentioned by Nissim (5). The only significant change in organ weights was an increase in weight of seminal vesicles in the 0.1% group with no change in testicular weight.

Additional data were obtained from adult ABCA mice used in experiments assaying the gonadotropic content of pituitary glands. Thirty-three mice on 0.2% nidroxyzone for two weeks showed decreases of 52.0% in weights of testes, of 34.2% in weights of seminal vesicles, and of 7.5% in

body weights compared with 34 controls. Fifteen mice on 0.3% nidroxyzone for two weeks had testicular weights inhibited by 55.2%, weight of seminal vesicles inhibited by 41.9%, and body weight by 15.5% (14 controls).

Attempts to prevent testicular atrophy with PMS or with pituitary homogenates were unsuccessful. The last experiment in Table 3 shows the effects of 2 I.U. of PMS administered three times a week for two weeks to five to six week old ZBC mice on 0.1% nidroxyzone. PMS did not alter testicular weights of either control or treated mice but did stimulate secretion of androgen in both groups as noted by increased weights of seminal vesicles. Even a dose of 10 I.U. of PMS daily, six times a week for two weeks, did not prevent testicular atrophy caused by 0.2% nidroxyzone in ZAF_1 adult mice. Testicular weights were 558 ± 32 mg.% in 10 control mice, 637 ± 25 mg.% in 9 controls given PMS, 263 ± 19 mg.% in 10 mice on 0.3% nidroxyzone and 247 ± 8 mg.% in 9 mice on 0.3% nidroxyzone plus PMS. Neither did the injection of pituitary homogenates in daily doses of one-half pituitary per mouse for two weeks prevent testicular atrophy in adult ABCA mice: testes were decreased in weight by 55.6% in 18 mice on 0.2% nidroxyzone and by 54.5% in 18 mice on 0.2% nidroxyzone plus pituitary injections, compared with 20 control mice.

Also, cysteine appeared to be unable to prevent the testicular effects of nidroxyzone in mice. In another experiment, not recorded in the table, the weights of testes and seminal vesicles, as mg.%, were decreased by 55 and 42% respectively in mice on nidroxyzone alone, and by 56 and 32% respectively in mice on nidroxyzone plus 1.0% cysteine (15 and 14 mice per group respectively).

TABLE 3. EFFECT OF NIDROXYZONE IN MALE MICE

Organ weights recorded as mg. % ±S.E. in first three experiments and as actual weight in mg. in last experiment.

Strain of mice	Age of mice	Days on exp.	Treatment	No. mice	Body wt. gm.		Organ weights			
					Initial	Final	Testes	Sem. ves.	Adrenals	Pituitary
C3H	21-22 da.	10	Controls	10	7.4	12.6	67.6±2.6	13.7±1.8	3.0±0.1	1.1±0.1
			0.1% nidrox.	10	7.4	11.8	34.3±1.0*	11.6±1.7	2.6±0.2	0.9±0.04
			21 da. old controls	10	7.4	—	32.7±1.5	4.2±0.6	2.2±0.1	0.6±0.04
C3H	7-9 mos.	21	Controls	10	27.1	27.5	481±18	528±47	16.5±0.9	6.1±0.4
			0.2% nidrox.	9	26.5	23.6	203±3*	410±21***	14.8±0.8	6.2±0.6
			0.1% nidrox.	10	27.1	25.3	218±6*	496±20	14.1±0.7	4.9±0.6
			0.05% nidrox.	10	26.3	26.1	517±12	511±27	14.5±0.6	5.7±0.3
ABCA	2-3 mos.	14	Controls	10	30.6	32.6	627±58	488±30	—	—
			0.1% nidrox.	10	32.3	32.4	746±37	610±32**	—	—
			0.05% nidrox.	10	31.6	30.9	827±33	567±31	—	—
			0.025% nidrox.	9	32.4	33.9	686±24	557±37	—	—
			0.01% nidrox.	10	32.1	33.9	552±78	520±40	—	—
ZBC	5-6 wks.	14	Controls	9	15.3	20.9	141.8	62.9	4.9	2.4
			Controls+PMS[a]	9	15.1	21.7	142.2	115.7	5.1	2.2
			0.1% nidrox.	8	16.9	22.2	85.6	64.9	5.0	2.4
			0.1% nidrox.+PMS[a]	10	16.5	20.4	71.5	108.5	4.5	3.1

* P=<.01.
** P=<.02.
*** P=<.05.
[a] Mice were injected subcutaneously with 2 I.U. PMS three times a week. Mice not receiving PMS were given saline subcutaneously.

TABLE 4. GONADOTROPIC CONTENT OF PITUITARY GLANDS OF INTACT AND CASTRATED ABCA MALE MICE THREE MONTHS OLD, ON 0.2% NIDROXYZONE FOR TWO WEEKS STARTING DAY OF CASTRATION

Pituitary glands assayed in 21- to 22-day old ABCA female mice, one-third pituitary gland per day for three days.

	Body wt. of donors (gm.)		No. recipient mice	Body wt. (gm.)	Uter.+vag. as mg.% ±S.E.*
	Initial	Final			
Saline controls	—	—	10	12.4	264 ± 18
Intact controls	34.6	37.3	11	12.4	591 ± 40
Intact treated	31.5	30.5	11	12.5	569 ± 42
Castr. controls	31.3	31.1	12	12.2	519 ± 35
Castr. treated	31.7	28.9	12	12.6	569 ± 25

* There is no significant difference between any of the last four groups.

Castrated male mice

C_3H male mice, two to five months old, were castrated and put on therapy for two weeks starting the day of castration. The weights of seminal vesicles were 105.7 ±5.3 mg. in 15 intact control mice, 15.3 ±1.2 mg. in 9 castrated controls, 19.9 ±2.1 mg. in 12 castrated mice on 0.1% nidroxyzone and 17.1 ±0.05 mg. in 12 castrated mice on 0.05% nidroxyzone. Thus, nidroxyzone had no effect on the weights of seminal vesicles of castrated mice either in doses causing testicular atrophy in intact mice (0.1%) or in doses too low to affect testicular weight in intact mice (0.05%).

Assay of pituitary glands

Four experiments assaying the gonadotropic content of pituitary glands of two- to three-month old ABCA male mice on nidroxyzone for two weeks were done. The results of two experiments with intact mice on 0.2% drug were equivocal, one suggesting an increase (10 to 11 recipient mice per group, P = >.1), the other suggesting a decrease in gonadotropic content (7 to 10 recipient mice per group, P = >.1). A third experiment with 0.3% drug in intact mice showed an insignificant decrease in gonadotropic content (11 to 12 recipient mice per group, P = >.1). Results of a fourth experiment in which pituitary glands of both intact and castrated mice were assayed are shown in Table 4. No difference in gonadotropic content between control or treated, intact or castrated mice was found. It is concluded that nidroxyzone did not affect gonadotropic content of pituitary glands of male mice.

The pituitary glands of treated and control female mice (ABCA mice in Table 2) were also assayed for gonadotropic content. The weights of uterus plus vagina of recipient mice were increased by 37.8% with control pituitary homogenates (P = <.01, 12 mice), by 35.6% with 0.2% nidroxyzone-treated pituitaries (P = <.01, 8 mice), and by 18.3% with 0.3% nidroxyzone-treated pituitaries (P = >.1, 11 mice). Though this increase of only 18.3% was not significantly different from the saline-injected

controls (12 mice), neither was it significantly different from the 37.8% increase produced by the injection of control pituitaries. Therefore it is concluded that nidroxyzone did not have any clear-cut effect on the gonadotropic content of pituitary glands of female mice.

DISCUSSION

It has been repeatedly shown that nidroxyzone induces testicular atrophy in rats (1, 2, 7). Nissim (5) found a similar effect in mice with nitrofurazone, which the present work confirms for nidroxyzone. The principal action of the nitrofurans on male sex organs appears to be on the seminiferous tubules resulting in spermatogenic arrest. Moderate doses appear to have little or no effect on interstitial cells or on androgen production in rats (1, 2). Nissim, however, noted a definite decrease in weights of seminal vesicles of mice with doses too low to affect testes (0.04% nitrofurazone by DD, 36 mice). With higher doses (0.15 to 0.3% nitrofurazone, 37 mice) a decrease of 59% in the weight of testes was obtained with no change in the average weight of seminal vesicles. However, with these higher doses he not infrequently observed mice with unusually large seminal vesicles associated with hypertrophy and hyperplasia of interstitial cells plus sufficient other mice showing decreases in weights of seminal vesicles to give an average that was similar to the controls. In hypophysectomized mice the high doses caused a decrease of approximately 33% in weights of seminal vesicles and of 45% in weights of testes. As a result of his findings Nissim suggests that low doses of nitrofurazone exert a direct anti-androgenic action but that high doses have a secondary androgenic effect due to an increase in gonadotropic activity of the pituitary gland. He thinks this increased gonadotropic action is the result of withdrawal of some substance, normally secreted by seminiferous tubules, which inhibits pituitary function, thus explaining his results on the basis of the inhibition theory proposed as the cause of increased gonadotropin after castration.

In the present work low doses of nidroxyzone showed no effect on weights of seminal vesicles and high doses tended to inhibit them. Therefore these results do not uphold Nissim's contention of a direct anti-androgenic effect with low doses and of a secondary androgenic action of high doses. However, nitrofurazone was used by Nissim and nidroxyzone in the present work.

The experiments involving assay of pituitary glands indicate that nidroxyzone did not affect the gonadotropic content of pituitary glands. This is in contrast to the findings of Nelson and Steinberger (2) who reported an increase in gonadotropic content in rats. In relating pituitary effects to possible mechanisms of action it should be remembered that gonadotropic content of the pituitary gland offers little or no information regarding secretory activity.

Nelson and Steinberger (3) observed no changes in estrus cycles or in ovarian weights of female rats. The adult female mouse responds to nidroxyzone by inhibition of estrus cycles and by decrease in weights of sex organs. The induction of estrus and the stimulation of sex organs by PMS in treated animals suggests an interference with the secretion of gonadotropin. However, the lack of effect of nidroxyzone in five to six weeks old female mice, an age at which sex organs are rapidly increasing in weight, argues against a decrease in gonadotropic activity.

Both PMS and pituitary injections failed to prevent testicular atrophy caused by nidroxyzone in mice. A similar lack of effectiveness of gonadotropic factors was observed in rats (1, 2). One would expect the administration of gonadotropin to prevent testicular atrophy if such atrophy were due to lack of secretion of gonadotropin.

Nelson *et al.* (2) and Featherstone *et al.* (7) found that estrogenic inhibition of the pituitary seemed to protect the testes of rats from nidroxyzone effect as evidenced by recovery times of testicular impairment. Nelson *et al.* (8) further observed that PMS could prevent this protective action of estrogen. These observations suggest that nidroxyzone acts only in the presence of gonadotropin, probably interfering with its action on spermatogenesis. On the other hand a direct action of the nitrofurans on testes is suggested by the decrease of testicular weight in hypophysectomized mice (5) and by an interference with citrate formation in testicular tissue *in vitro* (9). However, in the latter case whatever gonadotropin was present in the testes at the time of sacrifice would probably still be present.

If one assumes that nidroxyzone acts by blocking gonadotropic action then it is apparent in the present work that 12 I.U. of PMS a week will overcome this block in female mice but that 60 I.U. of PMS a week will not overcome it in male mice. The more or less selective action of nidroxyzone on spermatogenesis in male rats suggests a possible preferential inhibition of FSH. The difference in PMS effect in the sexes might be explained on the basis of greater sensitivity of female gonads than of male gonads to the FSH activity present in PMS. The work of Simpson *et al.* (10) indicates that ovaries of hypophysectomized rats are considerably more sensitive to FSH than are testes.

A species difference in the action of nidroxyzone is suggested by the lack of an effect on female sex organs of rats but an inhibition of them in mice and by the ability of cysteine to antagonize the induced testicular atrophy in rats but not in mice. The inability of PMS to prevent testicular atrophy in treated male mice but its effectiveness in counteracting diestrus and sex organ inhibition in treated female mice may reflect a sex difference either in the action of or sensitivity to PMS or in the action of nidroxyzone.

Acknowledgments

The author is grateful to Theodora M. Danielson, Marvin S. Segal and Donna J. Hammond for their technical assistance at different times.

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INFLUENCE OF INSULIN ON MAINTENANCE AND SECRETORY STIMULATION OF MOUSE MAMMARY TISSUES BY HORMONES IN ORGAN-CULTURE[1]

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ABSTRACT

Mouse mammary tissues from late prelactating, early prelactating, and nonpregnant multiparous female C3H/Crgl mice were subjected to various hormones in organ-culture in a synthetic medium. The three types of tissue were maintained with the addition of insulin and cortisol, to some extent with insulin alone, and not at all with cortisol alone. The more differentiated the tissue in the direction of secretory activity, the greater was the need for cortisol in addition to insulin for maintenance *in vitro*. Following culture in insulin and cortisol, mammary tissues showed one of the following responses: (1) alveolar maintenance with loss of secretory activity, (2) maintenance of the initial histologic structure, or (3) reorganization of alveolar epithelium.

Addition of mammotropin and somatotropin to media containing insulin and cortisol maintained the secretory state of late prelactating tissue and stimulated early prelactating and nonpregnant tissues to secretory activity. The optimum concentrations of MH and STH producing secretory responses in early prelactating tissue were four times greater than those effective in late prelactating tissue. With early prelactating tissue, the minimum hormonal combination capable of initiating secretion *in vitro* was either mammotropin or somatotropin, in addition to insulin plus cortisol

ORGAN-CULTURES of mammary glands from embryonic (1), young virgin (2, 3), and adult pregnant mice (4–8) have been cultured in partly or entirely synthetic media, supplemented with hormones. The functional capacity of prelactating mouse mammary gland has been investigated both in cell-culture (9, 10) and in organ-culture (4–8). In organ-cultures, insulin and cortisol permitted survival of late prelactating mouse mammary tissue (6), whereas mammotropin and cortisol stimulated secretory activity (4, 7). However, only one of several mammotropin preparations used, in combination with cortisol, was effective in stimulating secretion or permitting explant maintenance *in vitro*.

The purpose of the present study was to investigate in further detail the effects of insulin on the maintenance of alveolar structure of adul

Received December 12, 1960.

[1] Aided by American Cancer Society Grant E-122A and by Cancer Research Fund of the University of California.

[2] Trainee under U. S. Public Health Service Training Grant CRT 5045.

mouse mammary tissue in organ-culture and to determine the role of insulin in the hormonal induction of secretory activity. Experiments were designed to examine the effects of varying hormone concentrations, of the omission or addition of hormones, and of the initial physiologic state of the tissue. The hormonal requirements *in vitro* are compared with those already established by *in vivo* studies. Three stages of normal mammary gland development were cultured: (1) late prelactating, (2) early prelactating, and (3) nonpregnant.

METHODS

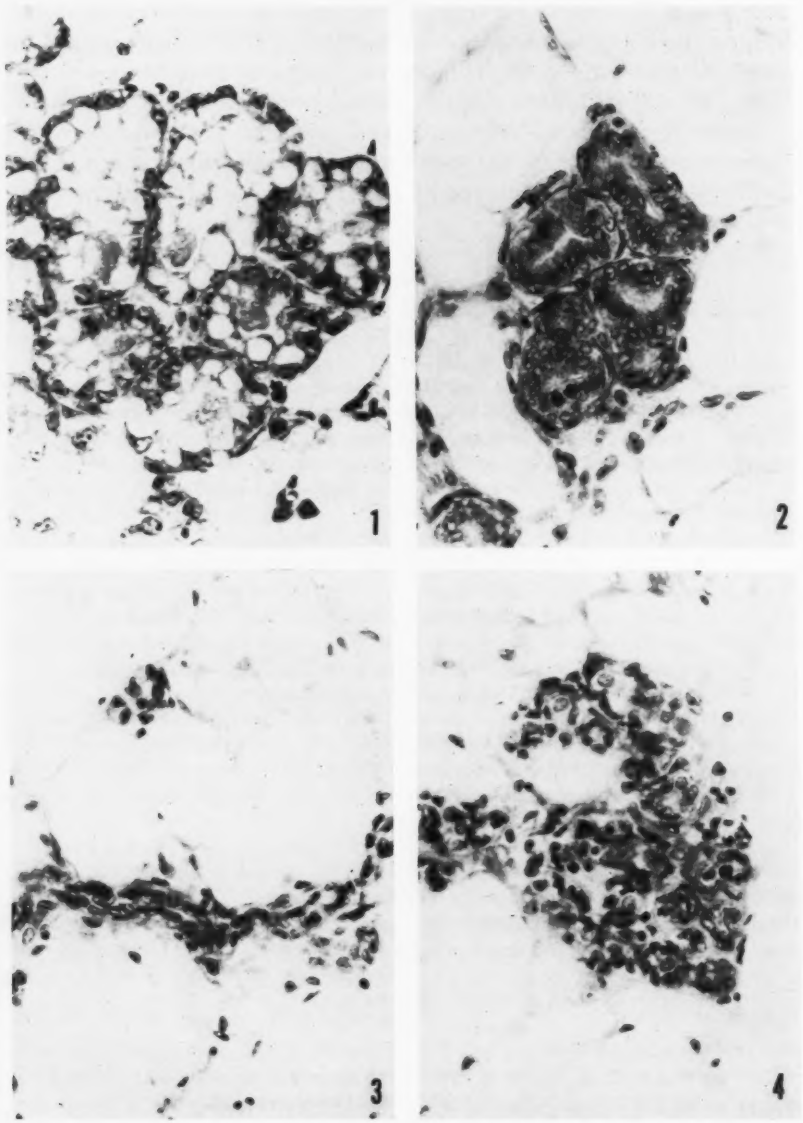
Tissues. Tissues were selected from mammary tumor agent-bearing, multiparous female C3H/Crgl mice. Explants were taken from mammary glands of animals between the 17th and 19th day of pregnancy (late prelactating), between the 10th and 12th day of pregnancy (early prelactating), and from animals at least two weeks after weaning (nonpregnant). The explants were about 1.5–2.0 mm. in diameter and about 0.5–1.0 mm. thick. Tissue samples were taken from areas adjacent to the tissues explanted for histologic determination of the initial state of the explant.

Hormones. The basal culture medium used was synthetic "199" (11). Amorphous insulin (I) (lot number W-3255, provided by Dr. Otto K. Behrens of Eli Lilly Company) was dissolved in 0.005N HCL and added to a measured volume of "199". This solution was sterilized by passage through a millipore filter with an average porosity of 0.45 μ. Aliquots of the filtrate were then added to the final culture medium in amounts required to yield the desired concentrations. Elias (12) found no significant increases in the dry weights of millipore filters after filtration of insulin solutions and concluded that no insulin was removed by this procedure. The insulin preparation contained 0.04% zinc and 2–3% glycagon, and had 20 units of activity/mg.

Ovine mammotropin (MH: lot numbers L2651 ASS, L2651 AP, and L2932 A; 25–30 I.U./mg.) and bovine somatotropin (STH: lot 57-1A) provided by Professor C. H. Li, were dissolved in a small volume of 0.0001 N NaOH, then added to measured volumes of "199". These hormone solutions were sterilized as above by passage through millipore filters.

Unesterified cortisol (F) (obtained from Merck, Sharp and Dohme) was dissolved in absolute ethanol. A small amount of the solution was added to a measured volume of "199". From this solution, aliquots were taken and added to the culture medium in amounts required to yield the desired concentrations. Control medium containing an ethanol concentration of 0.5% has no effect on mammary tissue (5). Mammary tissues used in the present study could tolerate concentrations of ethanol as high as 1.25%; however; the ethanol concentrations contained in most of the culture did not exceed 0.2%.

Culture Method. The culture method used was the Chen (13) adaptation to liquid media of the watch glass technic of Fell and Robinson (14), somewhat modified. The technic is described in a previous paper (7). In the present experiments, the incubator was humidified by placing pans containing distilled water on each shelf. A mixture of 95% O_2 – 5% CO_2 was bubbled through distilled water in the incubator at the rate of 0.5 to 0.7 liters per minute. By this method, the pH of the medium was maintained at about 7.4 to 7.6. The medium was removed on the third day and replaced with fresh medium, and the cultures were terminated at the end of five days. At the time of termination, the explants were observed grossly under the dissecting microscope and then transferred to Bouin's fixative. Serial paraffin sections of the tissues were made at 7μ and stained with modified Masson's stain.



All tissues were stained with Masson's stain and photographed at 425×.

FIG. 1. Prelactating lobule of mammary gland from 17 to 19-day pregnant mouse; late prelactating development. Note intracellular vacuolation, distended alveoli, and stainable secretion in alveolar lumina.

FIG. 2. Prelactating lobule of 10 to 12-day pregnant mouse; early prelactating development. Note absence of intracellular vacuolation, absence of stainable secretion in lumina, small alveoli, mitotic figure at bottom left.

Grading of Responses to Culture Media. Following culture, the serial sections of each explant were examined, and the responses of the *alveoli* were graded according to the following histologic criteria:

A. *Maintenance of alveolar structure:*
  −less than 50% of alveolar structure maintained
  +more than 50% of alveolar structure maintained

B. *Secretory activity of maintained alveoli:*
  −most alveoli small; little or no stainable secretion in the lumina; few cells with vacuoles
  +alveoli moderately distended; small amounts of stainable, serous-appearing secretion in the lumina; moderate number of cells with vacuoles
  ++alveoli greatly distended; large amounts of stainable, colloid-appearing secretion in the lumina; most cells with vacuoles

RESULTS

1. *Histology of tissues at time of explantation*

In the actively secretory, *late prelactating* lobules, the alveoli were distended, sometimes confluent, and showed moderate amounts of stainable secretion in the lumina. The alveolar epithelium was highly vacuolated (Fig. 1). The alveoli of the *early prelactating* lobules were small, with small lumina and usually little or no secretion. Vacuolation of the alveolar epithelium was generally absent (Fig. 2). *Nonpregnant* tissues showed alveolar clusters in different stages of regression (Figs. 3 and 4) or mostly ductal elements with relatively few alveolar clusters. The lumina of the alveoli were not readily seen. Variable numbers of unorganized, dark-staining cells were found scattered between alveoli. They appeared largely to be alveolar cells undergoing regression (Fig. 4).

2. *Response to insulin alone and in combination with cortisol (Table 1)*

Late prelactating explants showed extensive breakdown of alveolar structure following culture in medium containing insulin alone at concentrations of 5 μg/ml. Maintenance of alevolar structure was obtained after addition of cortisol to the above medium in concentrations ranging from 1 to 50 μg/ml. Insulin concentrations of 25 and 100 μg/ml. in combination with 8 μg/ml. of cortisol permitted 100% explant survival.

Although the alveolar structure of late prelactating tissue was well maintained in appropriate media, there was a definite loss of secretory activity. The alveolar cells showed a loss of the initially vacuolated state

FIG. 3. Section through mammary gland of multiparous female two weeks after weaning. Note regressed terminal duct and single alveolus at upper left.

FIG. 4. Section through mammary gland of multiparous female two weeks after weaning, showing cluster of incompletely regressed alveoli.

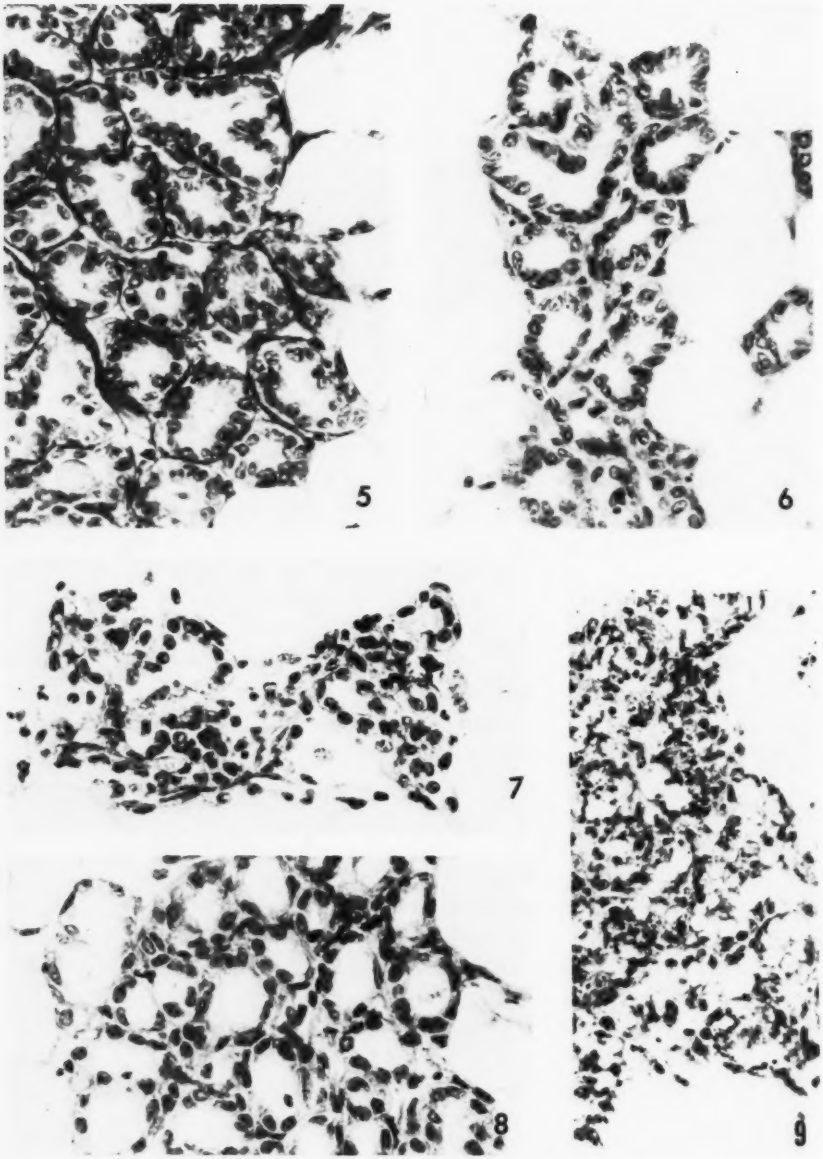


FIG. 5. Late prelactating lobular explant after 5 days in medium containing insulin plus cortisol (100+8 μg/ml.). Note inactive secretory appearance. *cf.* Fig. 1: loss of vacuolation and reduction in size of alveoli.

FIG. 6. Early prelactating lobular explant after 5 days in medium containing insulin plus cortisol (100+8 μg/ml.). Note inactive secretory appearance, maintenance of initial histology (*cf.* Fig. 2).

TABLE 1. RESPONSES OF MOUSE MAMMARY TISSUES TO INSULIN AND CORTISOL IN ORGAN-CULTURE

Culture medium (μg/ml.) I	F	Number of explants showing response indicated*								
		Late prelactating			Early prelactating			Nonpregnant		
		No. of explants	Maintenance alveolar structure†		No. of explants	Maintenance of alveolar structure†		No. of explants	Maintenance of alveolar structure†	
			−	+		−	+		−	+
0	0	8	8		8	8		8	8	
	8	8	8		8	8		8	8	
50					8	8		8		8
5		8	8		8	5	3	8		8
5	1	8	2	6	8	5	3	8		8
5	5	4		4	8	2	6	8		8
5	25	8	1	7	7	1	6	7		7
5	50	6	2	4	8		8	8		8
25	8	8		8	8		8	8		8
50	8				7	1	6			
100	8	8		8	16		16	8		8

* See text for explanation of symbols.
† Little or no stainable secretory material was observed in any of these explants.

(compare Figs. 1 and 5), and there was reduction of the stainable secretion in the alveolar lumina. Many of the alveoli also appeared to be smaller after explantation. Thus, insulin+cortisol-supplemented media permitted alveolar maintenance of late prelactating tissue for a period of 5 days, but the tissues resembled those from a less advanced stage of pregnancy.

Early prelactating explants retained their alveolar integrity to some extent in medium containing 5 μg/ml. of insulin alone. However, early breakdown was found in these explants. A definite improvement in alveolar structure resulted after cultivation in the presence of insulin concentrations of 5 μg/ml. supplemented with cortisol (5 to 50 μg/ml.). Explant structure was generally poor in medium containing 50 μg/ml. of insulin, but addition of 8 μg/ml. of cortisol provided a successful medium for alveolar maintenance. Cortisol alone (8 μg/ml.) was ineffective. Alveoli also were maintained by exposure to insulin doses of 25 and 100 μg/ml. in combination with 8 μg/ml. of cortisol.

FIG. 7. Nonpregnant explant after 5 days in medium containing insulin plus cortisol (50+5 μg/ml.). Note maintenance of regressed picture.

FIG. 8. Nonpregnant explant after 5 days in medium containing insulin plus cortisol (25+8 μg/ml.). (*cf.* Fig. 7.) Epithelial components are reorganized into moderately dilated alveoli.

FIG. 9. Late prelactating lobular explant after 5 days in medium "199" without added hormones. Note breakdown of alveolar structure and degeneration of vacuolated alveolar epithelium.

Alveolar structure was not only maintained in the presence of insulin+cortisol-supplemented media, but unlike late prelactating explants, the initial appearance of the alveolar cells of early prelactating explants was retained (compare Figs. 2 and 6). The alveoli usually remained small, the alveolar cells were non-vacuolated, and secretory material was either absent or present in small amounts.

Nonpregnant explants were maintained after culture in all of the insulin and cortisol combinations listed and even in the presence of insulin alone (5 and 50 μg/ml.). Following culture, the initial picture of epithelial involution was either retained without much alteration (Fig. 7), or alterations in structural organization were evident. In the latter instance, the alveoli were reorganized from "collapsed" forms into round and moderately dilated structures (compare Figs. 4 and 8).

Small amounts of serous-appearing secretion were present in some of the alveolar lumina. However, prior to explantation, some of the large ducts and alveoli contained finely granular secretory material. After cultivation, some of the reformed alveolar clusters superficially resembled the lobuloalveolar structure characteristic of early prelactating tissue, but there were some differences in detail. The number of cells per alveolus of nonpregnant explants appeared to be fewer than in early prelactating explants (compare Figs. 8 and 6). In addition, the alveolar epithelium of nonpregnant explants tended to be flatter than in early prelactating explants, the latter possessing low columnar epithelium.

All tissues showed extensive breakdown of alveolar structure and widespread necrosis in basal synthetic medium "199" without added hormones (Fig. 9). However, despite the histologic picture, explants of normal lobules cultured in the absence of hormones still contain enough viable cells to allow outgrowth when transplanted into gland-free mammary fat pads (15).

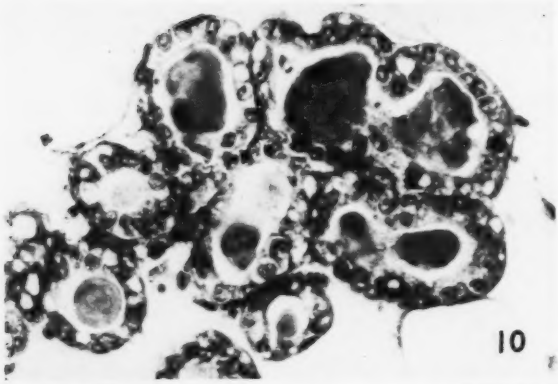
3. *Response to pituitary hormones in combination with insulin and cortisol (Table 2)*

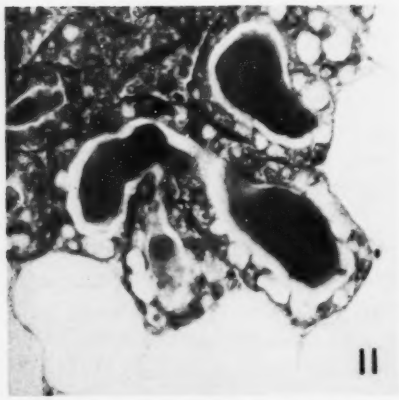
Late prelactating explants, after culture in medium containing I+F+MH+STH (100+8+50+50 μg/ml., respectively), showed not only alveolar maintenance, but also preservation of the secretory state existing prior to explantation. The alveoli were highly vacuolated and often confluent; the stainable colloid-appearing secretion in the lumina was abundant and appeared to be more dense than in the initial samples (compare Figs. 1 and 10). Some degree of secretory activity occurred when the dose of MH+STH was increased to 100+100 and 140+140 μg/ml., but the alveolar structure was poor. Omission of insulin or of cortisol from the 4-hormone combination resulted in alveolar breakdown. These data support the preceding findings that both insulin and cortisol were minimum requirements for explant integrity.

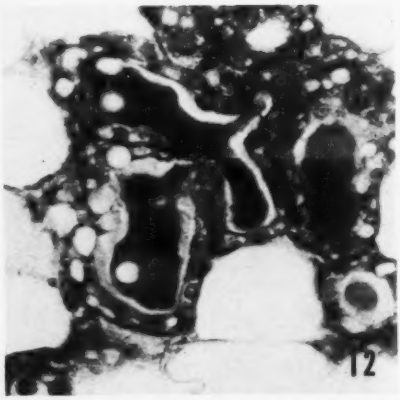
TABLE 2. RESPONSES OF MOUSE MAMMARY TISSUES TO PITUITARY HORMONES IN THE PRESENCE OF INSULIN, CORTISOL, AND BOTH INSULIN AND CORTISOL IN ORGAN-CULTURE

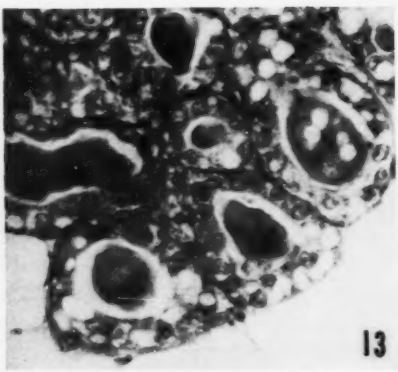
Culture medium (μg/ml.)				Number of explants showing response indicated*													
				Late prelactating					Early prelactating				Nonpregnant				
				No. of explants	Maintenance of alveolar structure		Secretory activity		No. of explants	Maintenance of alveolar structure		Secretory activity		No. of explants	Maintenance of alveolar structure		Secretory activity	
I	+ F	+ MH	+ STH		−	+	−	+ ++		−	+	−	+ ++		−	+	−	+ ++
	8	50	50	8	8		8											
	8	200	200						8	8		8						
	16	140	140											8	8		8	
100		50	50	8	8		8											
100		200	200						8	8		8						
100		140	140											8	8		8	
100	8	50	50	8		8		8										
100	8	100	100	4	2	2	2	2	4	3	1	2	2					
100	8	140	140	8	3	5	4	4	4		4	1	3					
100	16	140	140						6		6	1	5	14	1	13	13	1
100	8	200	200						24		24		24					
100	8	240	240						8	8		5	3					
100	8	200							16	1	15		16					
100	8		200						16		16		16					

* See text for explanation of symbols.









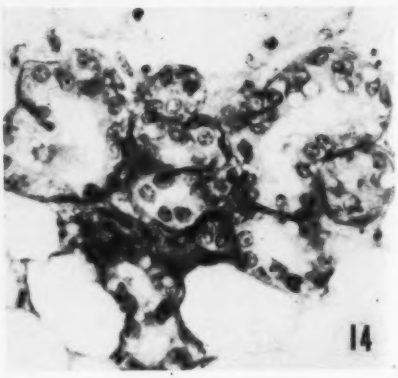


FIG. 10. Late prelactating lobular explant after 5 days in medium containing I+F +MH+STH. Note active secretory appearance.

FIG. 11. Early prelactating lobular explant after 5 days in medium containing I+F+MH. Note active secretory appearance.

Thus, "organotypic" maintenance of the advanced secretory state was obtained in the presence of mammotropin, somatotropin, insulin, and cortisol. Although the secretion in the alveolar lumina appeared to be more abundant after exposure to the 4-hormone combination, there was no unequivocal indication that secretory activity was advanced beyond the initial level.

Early prelactating tissue cultured in the presence of I+F+MH+STH (100+8+100+100 μg/ml., respectively) showed evidences of secretory responses, but the alveolar structure was generally poor. By increasing the MH+STH concentrations to 140+140 and 200+200 μg/ml. and keeping the I+F concentrations at 100+8 μg/ml., respectively, alveolar structure was improved greatly. However, increasing the MH+STH concentrations further to 240+240 μg/ml. resulted in tissue breakdown. In another instance, cortisol was added at a concentration of 16 μg/ml. and supplemented with I+MH+STH (100+140+140 μg/ml., respectively). The two-fold increase in cortisol dosage had no marked effects on survival or tissue response.

In all the hormonal combinations permitting alveolar maintenance in early prelactating tissue, there was some stimulation of secretory activity. Intracellular vacuolation was evident; colloid-appearing secretion, predominantly acidophilic, was present in the alveolar lumina; the alveoli became greatly distended and often confluent. The concentrations at which the secretory response was most pronounced were I+F+MH+STH at 100+8+200+200 μg/ml., respectively. Furthermore, secretory activity was induced by either MH (200 μg/ml.) or STH (200 μg/ml.) in combination with I+F (100+8 μg/ml., respectively). It was not possible to distinguish between histologic sections of early prelactating tissue culture in I+F+MH (Fig. 11), in I+F+STH (Fig. 12), or in I+F+MH+STH (Fig. 13). Omission of insulin from any of these hormonal combinations or omission of cortisol from the 4-hormone combination resulted in tissue breakdown. Thus, stimulation of secretory activity in early prelactating tissue was obtained *in vitro* in the presence of a pituitary hormone plus insulin and cortisol. The minimum hormonal combination capable of

FIG. 12. Early prelactating lobular explant after 5 days in medium containing I+F+STH. Note active secretory appearance.

FIG. 13. Early prelactating lobular explant after 5 days in medium containing I+F+MH+STH. Figs. 10, 11, 12, and 13 show similar histologic development.

FIG. 14. Nonpregnant alveolar cluster after 5 days in medium containing I+F+MH+STH. Note moderate secretory activity and small amount of stainable secretion in alveolar lumina.

stimulating secretion was insulin plus cortisol plus *either* mammotropin or somatotropin.

Nonpregnant tissue was maintained and even stimulated to some secretory activity following culture in I+F+MH+STH (100+16+140+140 μg/ml., respectively). Tissue breakdown resulted when either insulin or cortisol was omitted from the 4-hormone combination.

Characteristic of the secretory response were the occurrence of many vacuolated alveolar cells and the distention of many of the alveoli. Secretory material in the alveolar lumina was present as stainable (predominantly basophilic) and serous-appearing material. The amount and density of the secretion did not approach that found in prelactating tissue following culture in the presence of pituitary hormones, insulin, and cortisol (Fig. 14). Nevertheless, some secretory activity was initiated in some nonpregnant tissue in culture.

DISCUSSION

The results of these studies indicate that the responses obtained in organ-cultures of mouse mammary tissue required the presence of insulin. The primary effect of insulin appeared to be the maintenance of alveolar structure for the 5-day culture period. Omission of insulin from hormonal combinations otherwise capable of producing responses resulted consistently in breakdown of alveolar structure, accompanied by widespread necrosis.

The *kinds* of responses obtained *in vitro* were dependent upon the initial state of the explanted tissue and upon the specific hormonal treatment.

Influence of Insulin and Cortisol

Maintenance of the initial histology of early prelactating explants by insulin and cortisol was a consistent finding. Explants at this stage provided particularly favorable material for determining whether additional hormonal supplementation would induce secretory development.

In nonpregnant explants the reorganization of the regressing parenchyma into alveolar clusters presented some difficulty in interpretation. It was not always clear whether or not growth processes were involved. We have described the response conservatively as "reorganization" rather than "growth" for the following reasons: (1) mitotic figures rarely were found, and (2) the number of cells per alveolus did not seem to be more numerous after culture. The general absence of stainable material in the lumina suggests that the dilatation of the alveoli may have resulted from increased water uptake. Insulin has been shown to increase the water content in organ-cultures of embryonic limb-bone rudiments of the chick (16). However, the possibility that some cell proliferation had occurred cannot be excluded, in view of previous observations that insulin has growth-promoting effects in cell-cultures (17–20) and in organ-cultures

21, 22). Insulin also acts synergistically with ovarian steroids to stimulate mammary gland growth in hypophysectomized-gonadectomized rats (23, 24).

The mechanism by which insulin permits alveolar maintenance of mammary tissue *in vitro* is not clear. The possible nutritive role of insulin in the protein-free medium, "199", was not supported by the experiments of Elias (5), who showed that the beneficial effects of insulin could not be derived from bovine serum albumin or chick nucleoprotein fraction.

The effects of insulin on glucose uptake in mouse mammary gland organ-cultures have been studied in this laboratory by Moretti (25). Measurements of the daily glucose depletion from the medium indicated that glucose uptake by normal mammary tissue was stimulated by dosages of insulin from 1 to 200 μg/ml. However, stimulation of glucose uptake may be only one of several factors involved in the maintenance of mouse mammary tissue by insulin *in vitro*.

The contribution of cortisol also must be considered. The findings that cortisol alone does not maintain structural integrity *in vitro*, that insulin alone does to some extent, but that explant integrity was most consistent in the presence of both hormones, suggest that cortisol enhances the maintaining effect of insulin. Doses of insulin which did not support alveolar structure could, in the presence of cortisol, maintain prelactating tissue. For example, 5 μg/ml. of insulin alone did not maintain the alveoli of late prelactating tissue, but the addition of 1 μg/ml. of cortisol improved alveolar structure, and the addition of 5 μg/ml. resulted in 100% alveolar maintenance (Table 2). The more differentiated the normal mammary tissue in the direction of secretion, the greater was the need for cortisol in addition to insulin for alveolar maintenance *in vitro*.

Our observations that insulin and cortisol were the minimum hormonal requirements for consistent alveolar maintenance of mammary tissue explants, regardless of initial state, are in agreement with the findings of Elias (6). He reported that survival of late prelactating mouse mammary tissue in the presence of insulin alone was not so good as in media containing both insulin and cortisol. Trowell (26) reported that late prelactating rat mammary tissue survived for 6 days in synthetic medium containing insulin as the only hormonal component. However, he reports the occurrence of necrotic areas and reduction in the amount of secretion. The use of graded doses of insulin and cortisol has provided us with combinations in which necrotic areas (especially central necrosis) are completely absent.

Influence of Pituitary Hormones plus Insulin and Cortisol

Whereas late prelactating explants responded to insulin, cortisol, and pituitary hormones in a manner best referred to as "organotypic maintenance," early prelactating explants were stimulated to the secretory level found in late prelactating tissue. However, the optimum concentra-

tions of MH and STH for this stimulation were 4 times greater than those required by late prelactating tissue. It appears, then, that tissues from advanced stages of pregnancy are more sensitive to pituitary hormones than are those from earlier stages, or that the hormonal requirements for stimulation are greater than those for maintenance of the stimulated state. The finding that either MH or STH plus insulin and cortisol stimulated secretion is in agreement with *in vivo* results, where either MH or STH plus F were the minimum hormones inducing secretion in properly prepared hypophysectomized-ovariectomized-adrenalectomized C3H/Crgl female mice (27–29). It appears, then, that adult C3H/Crgl mammary tissue is unable to distinguish purified ovine mammotropin from bovine somatotropin either *in vitro* or *in vivo* (27). The interchangeability of STH and MH is not indicated for the mammary gland of all mouse strains *in vivo* (30); at the moment it is established *in vitro* only for our C3H strain.

The observation by Elias and Rivera (7) that a single preparation of MH, along with cortisol, stimulated secretion *in vitro* where other MH preparations failed, remains to be explained. That two MH preparations, in the presence of F, which failed to stimulate or permit survival of prelactating tissue *in vitro*, could do so in the presence of insulin, suggests that the single effective MH preparation previously used (7) may have contained insulin-like activity. An insulin-like activity of somatotropin has been described (31), and it is possible that such activity may exist in certain mammotropin preparations.

Although the response of nonpregnant explants to pituitary hormones did not approach the level of secretory activity obtained in prelactating tissue, the results show that normal inactive tissue can be stimulated to some degree. Thus, in the presence of insulin and cortisol, secretory responses were induced in mammary epithelium *in vitro* by pituitary hormones. The absence of similar responses in tissue cultured with insulin and cortisol alone indicates that mammotropin and somatotropin were acting specifically as "prelactogenic" hormones (stimulating maximum secretion without formation of true milk). Furthermore, as seen in early prelactating tissue, the "prelactogenic" effect occurred with *either* mammotropin or somatotropin, in synergism with insulin and cortisol.

The failure of mammary tissues to retain their histologic structure when either insulin or cortisol was omitted from hormone combinations permitting secretory responses, indicates that MH and STH were not directly involved in structural maintenance. It seems, therefore, that insulin and cortisol provided the minimum requirements for maintenance of alveolar structure *in vitro* and thereby permitted the pituitary hormones to exert their influences. True lactation (the presence of white milk in the alveoli) was not seen in any of our experiments. Determination of the specific hormonal and/or nutritional requirements for lactogenesis *in vitro* has yet to be done.

Acknowledgments

We are indebted to Professor K. B. DeOme, Dr. J. J. Elias and Dr. S. Nandi for suggestions regarding this study, to Miss Lillian Pissott for microtechnical assistance, and to Mr. Victor Duran for the photomicrography.

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STEROID HYDROXYLATION IN HUMAN FETAL ADRENALS: FORMATION OF 16α-HYDROXYPROGESTERONE, 17-HYDROXYPROGESTERONE AND DEOXYCORTICOSTERONE[1]

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ABSTRACT

Slices and homogenates of human fetal adrenals incubated with progesterone-4-C^{14} were shown to possess the enzymes necessary for converting this to deoxycorticosterone, 17-hydroxyprogesterone, and 16α-hydroxyprogesterone. The amount of product formed depended on the age of the fetus and to some extent on the conditions of incubation. The experiments suggest that 21-hydroxylase appears later in development than 16- and 17-hydroxylases. The possibility that androstenedione may be formed early in gestation but not later in gestation is suggested by the experimental results. Similar incubations with acetate-2-C^{14}, mevalonic acid-2-C^{14} or cholesterol-4-C^{14} yielded no detectable radioactivity associated with the steroid carriers.

SEVERAL attempts have been made to determine whether the human fetal adrenal possesses the enzymes necessary for the formation of the steroid hormones. Extraction of the adrenal glands has yielded materials with the chromatographic characteristics of androstenedione,[3] 11β-hy-

Received January 3, 1961.

[1] This work was supported by grants from the Association for the Aid of Crippled Children, the National Cancer Institute, U. S. Public Health Service (Grants: H 1529, A 808, HPS 5139), the American Cancer Society, Inc., the Jane Coffin Childs Memorial Fund for Medical Research, and the Charles and Marjorie King Fund. This is Publication Number 1043 of the Cancer Commission of Harvard University.

[2] Permanent Faculty Fellow of the American Cancer Society.

[3] Abbreviations and trivial names used refer to the following chemical substances: androstenedione (4-androstene-3,17-dione), 11β-hydroxyandrostenedione (11β-hydroxy-4-androstene-3,17-dione), dehydroepiandrosterone (3β-hydroxy-5-androstene-17-one), 17-hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione), deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione), corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), cortisol (11β-17,21-trihydroxy-4-pregnene-3,20-dione), Reichstein's Substance S (17,21-dihydroxy-4-pregnene-3,20-dione), 16α-hydroxyprogesterone (16α-hydroxy-4-pregnene-3,20-dione), mevalonic acid (β, δ-dihydroxy-β-methylvaleric acid), TPN (triphosphopyridine nucleotide).

droxyandrostenedione and dehydroepiandrosterone (1). Solomon and Lanman (2) pooled materials from the incubation of many fetal adrenals and established the identity of 17-hydroxyprogesterone and androstenedione as products of progesterone-4-C^{14} metabolism. Our own efforts have been restricted to the incubation of individual fetal adrenals with both non-radioactive and radioactive steroid substrates and the analysis of the products by chemical and physical means. We have been concerned with the development of the steroid enzyme systems as gestation proceeds and the effects of varying incubation procedures on the patterns of steroid synthesis.

The previous paper in this series (3) reported experiments in which unlabeled steroid substrates were used. The results obtained indicated that the 17, 21, and 11 steroid hydroxylases are present in the 15 week fetal adrenal. There was no evidence of 3β-ol steroid dehydrogenase activity. The present report concerns experiments in which C-14 labeled substrates were used to define further the enzymatic characteristics of this tissue and the changes that may occur during development.

MATERIALS AND METHODS

Apparatus.—A Gilson Volumetric fraction collector was used to collect samples from the partition column. Samples were counted at infinite thinness in a thin-window gas flow proportional counter (Nuclear Chicago). The counting was continued until enough counts had accumulated to reduce the probable error of counting to less than 3%. Tritium was counted in the proportional counter with the window removed. Ultraviolet absorption of the samples was determined in a Beckman DU spectrophotometer comparing the experimental readings from 220 mμ to 260 mμ with those of standard solutions of the steroids at these wavelengths.

Countercurrent distributions were performed on either the 25 tube all-steel or the 50 tube all-glass machine made by H. O. Post Scientific Instrument Co., New York. Infra-red analysis of recrystallized derivatives was done on the Perkin Elmer Infra-red Spectrophotometer. A Rinco rotating still was used to evaporate all organic solutions under vacuum.

Solvents.—All organic solvents were redistilled by and obtained from R. J. Curtis, Chemistry Laboratory, M.I.T., Cambridge, Mass. Pyridine and acetic anhydride were redistilled immediately before use.

Reagents.—The following radioactive substrates were used: progesterone-4-C^{14}, (30.7 μC/mg.), cholesterol-4-C^{14} (16.5 μC/mg.), mevalonic acid-2-C^{14} (1.94 μC/μmole), and acetate-2-C^{14} (0.56 μC/mg.). The acetate-2-C^{14} was obtained from Nuclear Chicago Corp. The other radioactive substances were obtained from New England Nuclear Corp. TPN (triphosphopyridine nucleotide) and glucose-6-phosphate were obtained from Sigma Chemical Co. The steroids used in these experiments were generously supplied by the Upjohn Company.

Incubation Procedure.—Human fetuses were obtained at therapeutic interruptions of pregnancy and the adrenals and gonads were removed immediately and placed in cold saline. Within 20 minutes of the time of the operation, the tissue was homogenized in phosphate buffer, pH 7.4, containing, per ml., 20 micromoles K^+, 10 micromoles Mg^{++}, 20 micromoles Cl^-, and 40 micromoles of phosphate. Aliquots of the homogenized material equivalent to 20 to 400 mg. of adrenal tissue were transferred to incubation

vessels and one microcurie of progesterone-4-C^{14} dissolved in propylene glycol was added to the vessel. In certain experiments 2.7 micromoles of TPN and 24 micromoles of glucose-6-phosphate were added as a TPNH-generating system. Our previous experiments (3) had shown that the fetal adrenal has a very active glucose-6-phosphate dehydrogenase system.

The vessels were shaken in a constant temperature bath at 37° with air as the gas phase for periods of 1 to 4 hours. The incubation mixture was then removed and stored in the frozen state. In a few experiments, adrenal slices were incubated with progesterone-4-C^{14} in a $NaKPO_4$ buffer containing, per ml., 80 micromoles Na^+, 40 micromoles K^+, 100 micromoles Cl^-, and 40 micromoles phosphate.

Similar experiments were performed with adrenal homogenates using acetate-2-C^{14}, cholesterol-4-C^{14}, or mevalonic acid-2-C^{14} as substrate.

Extraction and Purification.—The following steroids were added to the incubation material to be extracted in amounts of 0.8 to 1.5 mg. each: androstenedione, deoxycorticosterone, 17-hydroxyprogesterone, 11β-hydroxyandrostenedione, corticosterone, and cortisol. The incubation mixture was extracted three times with five volumes of ethyl acetate, followed by three extractions with five volumes of acetone and three with five volumes of methanol. The extracts were pooled and evaporated to dryness and the residue was partitioned between hexane and 90% methanol 10% water, using a five funnel countercurrent distribution with stripping (4). Aliquots of the original extract and aliquots from hexane and methanol fractions of the partition were counted.

Chromatography.—The methanol fraction was dried and chromatographed on a Hyflo Supercel partition column (5). Ten gm. of washed Hyflo Supercel was blended with 6 ml. of lower (stationary) phase of the system: 2,2,4-trimethylpentane/90% methanol:10% water. Using upper (mobile) phase the Hydro Supercel was made into a slurry and manually packed into a glass column (0.8 cm. diameter) with a glass wool plug at the bottom. The packed column was equilibrated by allowing the mobile phase to drip through the column by gravity for a minimum of five hours before use. The rate was from three to five ml. per hour.

The dried methanol fraction from the hexane-90% methanol partition was dissolved in 0.18 ml. of the stationary phase of the system. Washed Hyflo Supercel (300 mg.) was added to the sample and the material carefully adsorbed on the Hyflo. A slurry was then made with mobile phase and the sample was transferred to the prepared column and manually packed on top of it. Mobile phase was allowed to run through the column and 1 ml. fractions were collected. After 100 fractions were collected, gradient elution was begun by introducing dichloroethane into the upper reservoir (5). Gradient elution was continued until an additional 300–350 fractions were collected.

The effluent fractions were allowed to evaporate in air at room temperature. The residue was dissolved in 5 ml. of absolute methanol and aliquots were taken for counting and for measurement of ultraviolet absorption.

To eliminate the possiblity that the radioactive progesterone used as substrate might be contaminated with other radioactive substances, a control experiment was performed in which 1 microcurie of progesterone-4-C^{14} and six steroid carriers, androstenedione, 17-hydroxyprogesterone, deoxycorticosterone, 11β-hydroxyandrostenedione, corticosterone, and cortisol, were carried through the entire extraction, purification, and chromatographic procedure. Every fourth tube from 0–400 was analyzed for absorbance at 240 mμ and for radioactivity. No radioactive peak was found except a single large peak in the progesterone region (tubes 40–60). The steroid carriers separated well from one another and were completely free of radioactivity. Estimates of recovery revealed that from 80–90% of the carrier material could be recovered from the column using the techniques described. There was no difference in the recoveries of individual steroids; however, the more polar compounds had broader, less well defined peaks than

the non-polar steroids. As biological controls, samples of fetal liver and fetal placenta were incubated with progesterone-4-C^{14} as described for the fetal adrenals. The same procedures of incubation, extraction, purification, and column chromatography were used and the elution patterns are shown in Figures 1 and 2. The elution patterns are distinctly different from those of the fetal adrenals, but the radioactive substances formed have not yet been identified.

RESULTS

The pattern of steroid synthesis in human fetal adrenals varies with the age of the fetus, the conditions of incubation (presence or absence of cofactors), and probably to some extent with the length of time of incubation.

Table 1 presents data on the maturity of the fetuses studied and the conditions of incubation. Specimens I–III were arbitrarily grouped as very young fetuses, specimens IV–VII as intermediate, and specimen VIII as an older fetus.

Specimens I and II were incubated in identical fashion and the incubation media pooled for analysis. Figure 3 shows the pattern of elution from

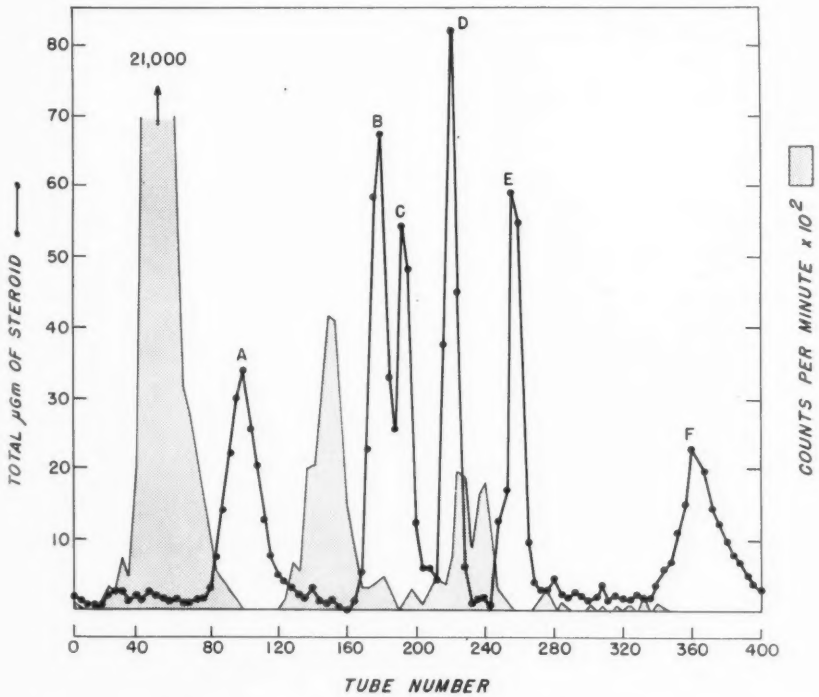

FIG. 1. Chromatogram—fetal liver. A. Androstenedione, B. Deoxycorticosterone, C. 17-Hydroxyprogesterone, D. 11β-Hydroxycorticosterone, E. Corticosterone, F. Cortisol.

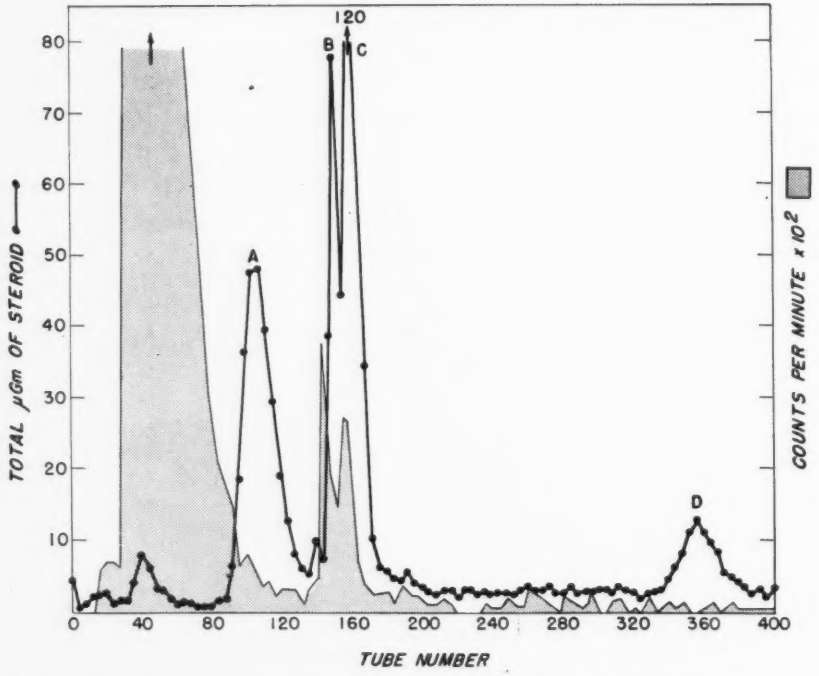

FIG. 2. Chromatogram—fetal placenta. A. Androstenedione, B. Deoxycorticosterone, C. 17-Hydroxyprogesterone, D. Cortisol.

TABLE 1. AGE AND CONDITIONS OF INCUBATIONS OF FETAL ADRENALS

Specimen (sex)	F.W. (gm.)	C.R. (cm.)	G.A. (wk.)	Incubation conditions		Time (hrs.)	Adr. wt. (mg.)	Tissue wt. (mg.)
I		4.0		*	+	4	30	30
II		5.5		*	+	4	20	20
III				†	+	4	25	25
IV (F)		10.0		*	+	4	215	108 in 2v.
V		10.5		†	−	3	244	126
VI (F)	50	10.0	12	*	−	1	118	118
VII (F)	75	10.0	13.5	*	−	3	278	278
VIII (M)	1385	25.5	25	*	−	1	3564	370

F.W. = Fetal weight
G.A. = Gestational Age
C.R. = Crown Rump Length
Adr. Wt. = Total weight of both adrenals
Tissue Wt. = Weight of adrenal tissue per incubation vessel
* = $KMgPO_4$ Buffer
† = $NaKPO_4$ Buffer
+ = TPN, Glucose-6-PO_4 added
− = No cofactor

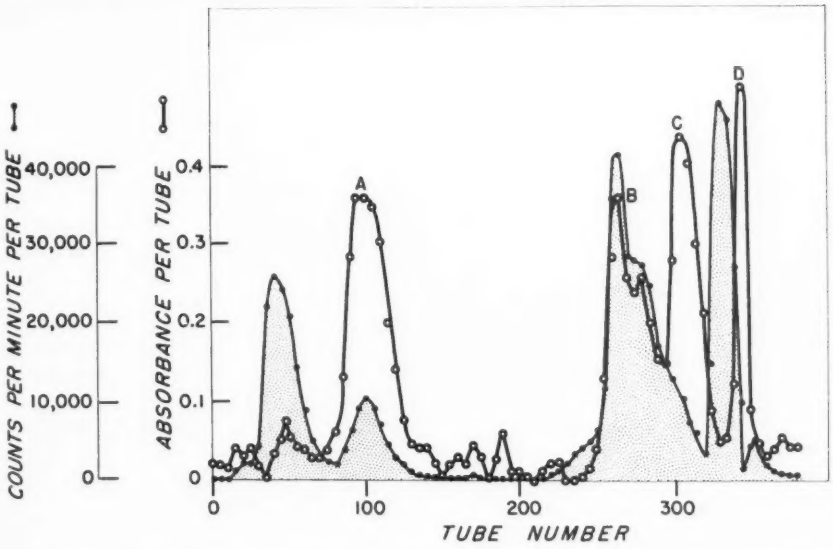

FIG. 3. Chromatogram—Specimens I and II. A. Androstenedione, B. 17-Hydroxyprogesterone, C. 11β-Hydroxyandrostenedione, D. Corticosterone. The radioactive peak preceding androstenedione is unaltered progesterone-4-C^{14}.

column partition chromatography of these pooled media. This was the only instance in which radioactivity appeared in the androstenedione region. The contents of tubes 90 to 110 were pooled and partitioned in a 24 transfer countercurrent distribution. The distribution curves for radioactivity and ultraviolet absorption at 240 mμ coincided and two crystallizations of the radioactive material from methanol showed constant specific activity. Though there is some evidence that the radioactive product is indeed androstenedione, complete proof of radiochemical purity has not been established.

One of the major radioactive materials produced by the early fetal adrenal is associated with the 17-hydroxyprogesterone added as carrier. A second major radioactive product was eluted between peaks C (11β-hydroxyandrostenedione) and D (corticosterone) and has been termed the X peak. Carrier cortisol (recovered by washing the column with methanol) had a small amount of radioactivity associated with it.

Extraction and chromatography of the incubation medium of adrenals from specimen III yielded the elution pattern shown in Figure 4. These adrenals had been minced and incubated in the $NaKPO_4$ buffer with added TPN and glucose-6-phosphate. In contrast with other incubations, only very small amounts of radioactive steroids were formed. The largest amount of radioactivity coincided with the 17-hydroxyprogesterone carrier. In addition, very small amounts of radioactivity appeared in the X region

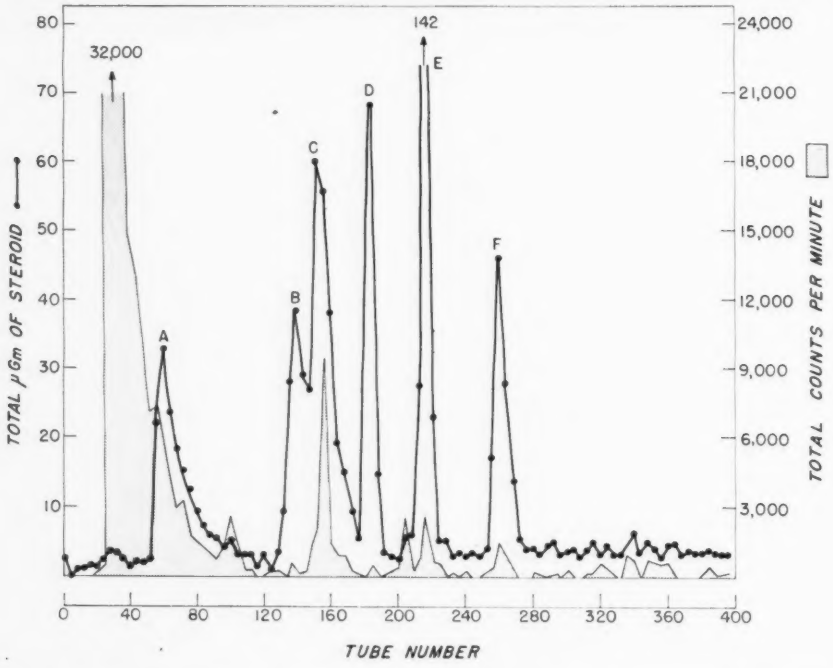

FIG. 4. Chromatogram—Specimen III. A. Androstenedione, B. Deoxycorticosterone, C. 17-Hydroxyprogesterone, D. 11β-Hydroxyandrostenedione, E. Corticosterone, F. Cortisol.

and in the corticosterone and cortisol regions. Because the progesterone and androstenedione peaks in this elution were unusually close together, it is impossible to determine whether a small amount of androstenedione might have been produced; however, it is clear that no large amount of the substance appeared.

These experiments provide evidence for the existence in the adrenal of the very young fetus of enzymes involved in steroid biosynthesis. In contrast to the older specimens, these adrenals from young fetuses may form androstenedione.

The intermediate group of fetuses provided an opportunity to compare changes in patterns of steroid synthesis with age, presence or absence of cofactors, intact vs. homogenized cells, and with length of incubation. Adrenals from specimens V and VII were treated in a comparable fashion except that the adrenal from V was sliced, leaving the cells intact, and the amount of adrenal tissue incubated was less (126 vs. 278 mg.). The elution pattern from the incubation of the homogenized adrenal (specimen VII) is given in Figure 5. Major peaks of radioactivity appear in the deoxycorticosterone, 17-hydroxyprogesterone, and X regions. No radio-

activity appeared in association with carrier cortisol. The elution pattern of specimen V differed from that of Figure 5 only in that no radioactivity appeared in the deoxycorticosterone region and a large amount of radioactivity appeared in the cortisol and corticosterone regions. Table 2 summarizes the amounts of radioactivity appearing under the respective peaks for the materials from specimens V and VII. These findings suggest that 11-hydroxylation requires either the addition of cofactors to a homogenate or the presence of the intact cell which can generate these cofactors.

Adrenals from specimens VI and VII were incubated in identical fashion except for: 1) length of time of incubation and 2) the amounts of adrenal tissue incubated (118 vs. 278 mg.). Figure 6 gives the results obtained on incubation of the adrenals from specimen VI. This pattern differs from that of Figure 5 only in that the radioactivity in the corticosterone region is a distinct peak, however, it does not coincide exactly with the carrier corticosterone. Since Reichstein's Substance S and corticosterone appear in the same region of the column, it is possible that this radioactive peak represents Substance S. Quantitative differences are evident in the 17-hydroxyprogesterone and X region (Table 2). However, when homogenized

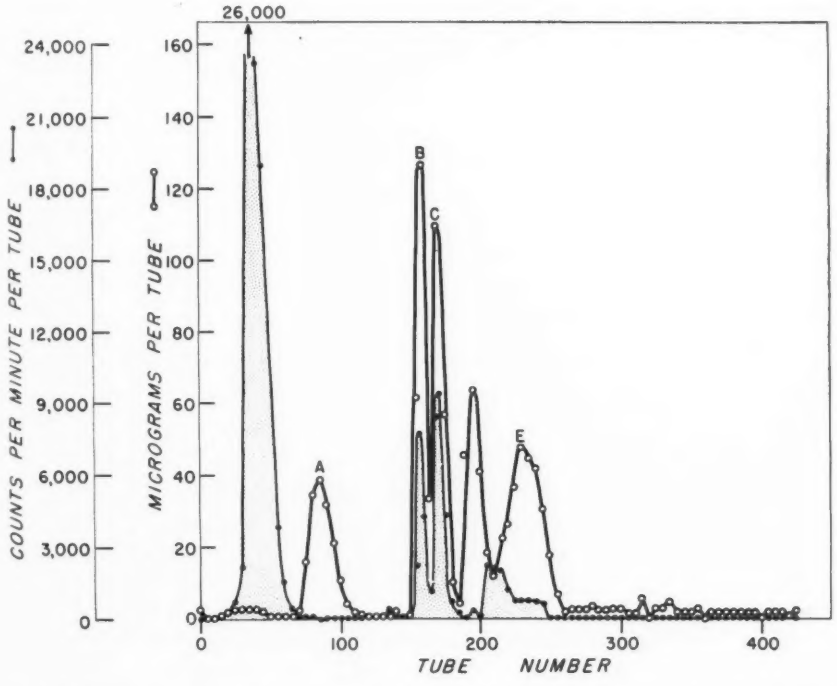

FIG. 5. Chromatogram—Specimen VII. A. Androstenedione, B. Deoxycorticosterone, C. 17-Hydroxyprogesterone, D. 11β-Hydroxyandrostenedione, E. Corticosterone.

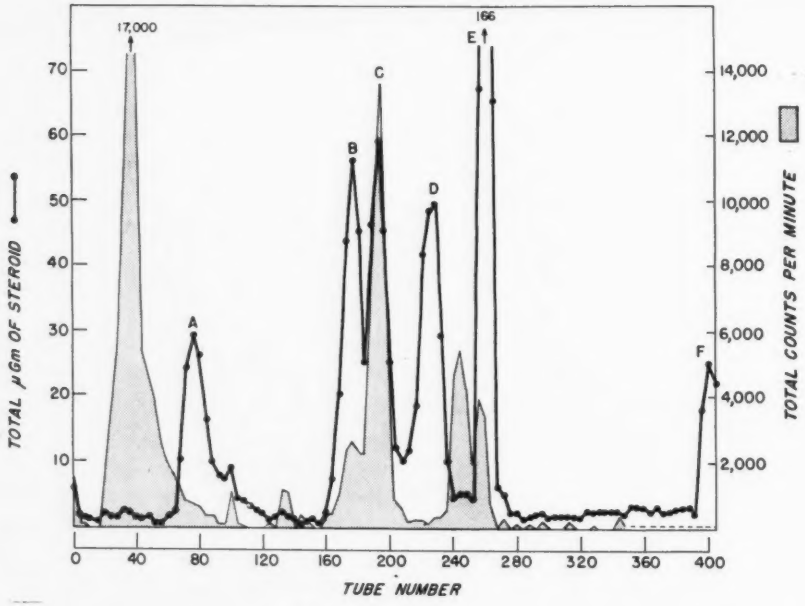

FIG. 6. Chromatogram—Specimen VI. A. Androstenedione, B. Deoxycorticosterone, C. 17-Hydroxyprogesterone, D. 11β-Hydroxyandrostenedione, E. Corticosterone, F. Cortisol.

tissue was incubated without added cofactors, no radioactivity appeared in the cortisol region.

Adrenals from specimens IV and VII were incubated similarly except that a TPNH-generating system was added to the former. Much less radioactivity appeared in the 17-hydroxyprogesterone area, and much more in the X, corticosterone, and cortisol regions of specimen IV (Table 2). The tissue from specimen IV was divided into two equal portions of 108 mg. each and incubated in separate vessels. Each vessel contained 1 microcurie of progesterone-4-C^{14} and added cofactors. After incubation the

TABLE 2. RADIOACTIVITY ASSOCIATED WITH ADDED STEROIDS, ADRENALS OF FETUSES AGED 10–15 WEEKS

Specimen	Total counts in region				
	Deoxycortico-sterone	17-hydroxypro-gesterone	X	Corticosterone	Cortisol
IV*	0	30,000	260,000	320,000	310,000
V	0	60,000	50,000	50,000	50,000
VI	38,000	153,000	65,700	0	0
VII	34,600	79,800	45,300	0	0

* These figures represent the results of the pooled material from two vessels incubated with 108 mg. adrenal tissue and 1 microcurie of progesterone-4-C^{14}.

vessels were pooled for extraction and chromatography. Although twice the usual amount of radioactivity was present in the beginning of the extraction, the amounts of radiocativity in the X, corticosterone and cortisol regions were much greater than can be explained by a simple doubling of the precursor.

The conditions of incubation of specimen IV were comparable to those of the younger fetal adrenals (I and II), since both had TPNH-generating systems. The lack of radioactivity in the androstenedione region in all the specimens of the intermediate group is in sharp contrast to the results found in the adrenals from younger fetuses.

The radioactive materials present in the deoxycorticosterone, 17-hydroxyprogesterone, and X regions were identified as follows:

Deoxycorticosterone

The contents of tubes 152–162 of the column from specimen VII were pooled (34,600 cpm). The pooled material was evaporated and partitioned in a 24 transfer countercurrent distribution, using the system 90% CCl_4: 10% $CHCl_3$/80% MeOH:20% H_2O. The amount of steroid, measured by ultraviolet absorption, and the radioactivity of the sample were plotted against tube number. Though there was good correspondence of weight and radioactivity, the spread of the values made it impossible to fit the experimental points to the theoretical normal distribution curve. This suggested that the sample was not pure. Tubes 6–18 of the distribution were pooled, 16.3 mg. of deoxycorticosterone were added as carrier, and the material was twice crystallized from methanol (Table 3). The final crystals were acetylated with redistilled acetic anhydride and pyridine. The specific activity of the derivative agreed with that of the recrystallized deoxycorticosterone. The infra-red spectrum of the deoxycorticosterone acetate was identical with that of an authentic sample of deoxycorticosterone acetate.

17-hydroxyprogesterone

The contents of tubes 168–178 of the column from specimen VII (Fig. 5) were pooled (79,800 cpm), and evaporated. The residue was partitioned in a 24 transfer countercurrent distribution in the system 70% methanol:30% H_2O/CCl_4. The approximation of the experimental points on the distribution curve with the theoretical curve of normal distribution by the method of Baggett and Engel (6) is shown in Figure 7. The partition coefficient for 17-hydroxyprogesterone in this system is 1.4, $\theta = 2.41$, and the average specific activity was 94,760. The plot of coded specific activity [(log S.A. -4) $\times 10^3$] against the tube number is shown just above this curve. An analysis of variance of the values within $\pm 2\theta$ cf the peak gave a variance ratio of 0.0908, which provides evidence for the radiochemical purity of the sample. The contents of tubes 10–18 of the distribution were

TABLE 3. PURIFICATION OF SUBSTANCES DERIVED FROM INCUBATIONS OF FETAL ADRENAL TISSUES
The figures represent specific activity expressed as cpm/mg.

Substance	Specimen	Crystallization I		Crystallization I		Crystallization III		Derivative	Crystallization of derivative
		Crystals	Mother liquor	Crystals	Mother liquor	Crystals	Mother liquor		
Deoxycorticosterone	VII	1520	1680	1240 (407 cpm/μmole)	1200			1294* (479 cpm/μmole)	
17-Hydroxyprogesterone	VII	1779	2354	2077	2817	2901 (951 cpm/μmole)	2518	2240† (646 cpm/μmole)	
Deoxycorticosterone	VIII	1080 (354 cpm/μmole)	1150					868* (321 cpm/μmole)	
17-Hydroxyprogesterone	VIII	1800	1990	1697 (557 cpm/μmole)	2400			1950† (563 cpm/μmole)	2030 (584 cpm/μmole)
16α-Hydroxyprogesterone	VIII	2840	1920	2620 (860 cpm/μmole)	2530			2770* (1030 cpm/μmole)	

* Acetate.
† Androstenedione.

pooled, diluted with 16.6 mg. of carrier 17-hydroxyprogesterone and the material crystallized three times from methanol (Table 3). The final crystals were oxidized with chromic acid and the steroid derivative was extracted with redistilled ethyl acetate. The extract was dried on a rotating still and chromatographed on a 5 gm. Hyflo Supercel column. The material eluted in the androstenedione region was evaporated and analyzed for ultraviolet absorption and for radioactivity. The specific activity of the derivative is shown in Table 3. Because of insufficient material it was impossible to recrystallize the androstenedione further. The infra-red spectrum of the material was determined and found to match that of authentic androstenedione.

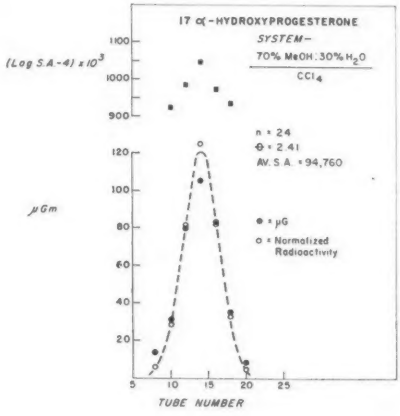

FIG. 7. Theoretical curve of normal distribution with experimental points. Normalized radioactivity represents counts per minute per mg. steroid divided by the average specific activity.

X region

The contents of tubes 201–219 of the column from specimen VII were pooled (36,000 cpm) and evaporated. A paper chromatogram was prepared with samples of 11β-hydroxyandrostenedione, 16α-hydroxyprogesterone, and corticosterone as standards. In the center a track was prepared with 0.04 mg. of pure 16α-hydroxyprogesterone and to this was added the material from the X region. Other experiments which will be reported later had indicated that the material in this region might be 16α-hydroxyprogesterone. The chromatogram was placed in the modified Bush system, ligroin:toluene 2:1/methanol:H_2O 7:3, and the moving phase of the system was allowed to drip off the edge of the paper (total chromatographic time, 16 hrs). 11β-Hydroxyandrostenedione, 16α-hydroxyprogesterone, and Reichstein's Substance S were 23.4, 15.4, and 11.0 cm. from the starting line respectively. Three ultraviolet-absorbing spots, corresponding to these three standards, appeared in the unknown track. This fraction of the column effluent would contain small amounts of substances from the adjacent peaks of 11β-hydroxyandrostenedione, and

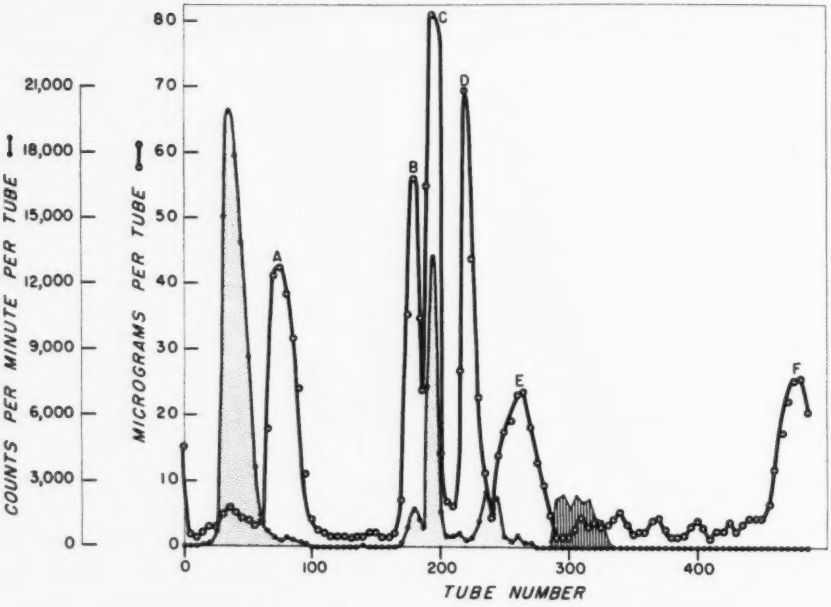

FIG. 8. Chromatogram—Specimen VIII. A. Androstenedione, B. Deoxycorticosterone, C. 17-Hydroxyprogesterone, D. 11β-Hydroxyandrostenedione, E. Corticosterone, F. Cortisol.

corticosterone. The 16α-hydroxyprogesterone spot was eluted with absolute methanol and counted. A total of 9,294 counts per minute was associated with this material. The spots of 11β-hydroxyandrostenedione and corticosterone were eluted similarly and counted, the former having 4,200 counts per minute and the latter having 28,000 counts per minute. The 16α-hydroxyprogesterone material was evaporated and 8.8 mg. of pure 16α-hydroxyprogesterone was added as carrier. Two crystallizations from methanol showed that the specific activity was constant and the crystals and mother liquors had the same specific activities (crystals, 1400 cpm /mg.; mother liquor, 1490 cpm/mg.).

These experiments provide evidence that the three substances deoxycorticosterone, 17-hydroxyprogesterone, and 16α-hydroxyprogesterone are formed in substantial quantities by the human fetal adrenal at least as early as 12 weeks gestational age. With intact adrenal cells or with the addition of a TPNH-generating sytem to homogenates, radioactivity appears in association with the carriers corticosterone and cortisol. It must be emphasized that corticosterone and Reichstein's Substance S (17-hydroxydeoxycorticosterone) appear in the same fractions of effluent from the column. Thus the radioactivity appearing with corticosterone may actually represent the formation of radioactive Substance S or a mixture of Substance S and corticosterone.

Specimen VIII was the oldest fetus studied. The two adrenals, weighing 3.56 gm., were homogenized in $KMgPO_4$ buffer and aliquots of the homogenate were incubated under various conditions. Aliquots incubated with progesterone or 17-hydroxyprogesterone yielded Porter-Silber chromogens (3). Figure 8 gives the results of incubating an aliquot of the homogenate with progesterone-4-C^{14} and analyzing the products by column chromatography. No cofactors were added, hence the absence of radioactivity in the cortisol and corticosterone regions is not surprising. Radioactivity appeared in the deoxycorticosterone, 17-hydroxyprogesterone, and 16α-hydroxyprogesterone regions. Tritiated aldosterone was added as a carrier to the material put on the column. The labeled aldosterone appeared in a peak just following corticosterone, but no C^{14} labeled material was found in this region. This is evidence that aldosterone was not synthesized by fetal adrenal homogenates in this experiment. The amount of radioactivity associated with deoxycorticosterone was distinctly less than that appearing in the 12 to 15 week fetal adrenals. The amount of 16α-hydroxyprogesterone would appear to be about the same. The radioactive materials were identified as follows:

Deoxycorticosterone

The contents of tubes 170–184 of the column were pooled and evaporated (20,176 cpm). The material was partitioned on a 24 transfer countercurrent distribution using the system 90% CCl_4:10% $CHCl_3$/80% MeOH: 20% H_2O. In Figure 9 experimental points and the theoretical normal distribution curve for DOC in this system are plotted against tube number. An analysis of variance of the values for the specific activities of these samples gave a variance ratio of 0.1716. This is evidence of radiochemical purity.

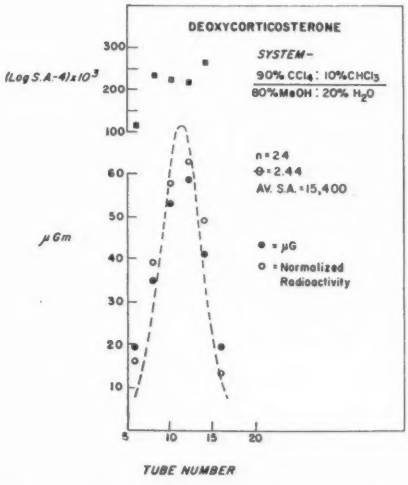

FIG. 9. Theoretical normal distribution curve with experimental points.

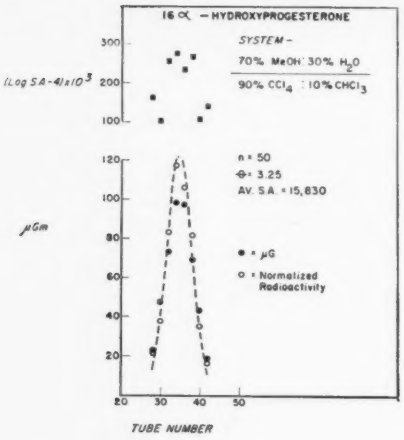

FIG. 10. Theoretical normal distribution curve with experimental points.

The materials in the peak area were pooled, 10.7 mg. of pure deoxycorticosterone was added and the mixture was crystallized from absolute methanol. The specific activities of the crystals and the mother liquor are recorded in Table 3. The crystals were acetylated and the specific activity of the acetate was determined. Both crystals of the deoxycorticosterone and its acetate had the same specific activity, and the infra-red spectrum of the acetate agreed with that of authentic deoxycorticosterone acetate.

17-hydroxyprogesterone

The contents of tubes 190–200 of the column from the experiment with specimen VIII were pooled and evaporated (98,370 cpm). The material was partitioned in a 50 transfer countercurrent distribution using the system 75% methanol:25% H_2O/CCl_4. The material in tubes 24–42 of the distribution was pooled and 33.8 mg. of pure 17-hydroxyprogesterone was added to the material. Table 3 presents the specific activities of the material recrystallized from absolute methanol. The final crystals were oxidized with chromic acid and the androstenedione separated as described previously. The specific activity (cpm/μmole) of the crystallized androstenedione agreed with that of the original 17-hydroxyprogesterone (Table 3). This provides additional evidence for identification of the radioactive product as 17-hydroxyprogesterone. The infra-red spectrum of the recrystallized androstenedione agreed with the spectrum of authentic androstenedione.

16α-hydroxyprogesterone

The contents of tubes 230–242 ("X" region) of the column were pooled and dried and 1 mg. of pure 16α-hydroxyprogesterone was added as carrier. The material was distributed over 50 transfers by the countercurrent

method using the system 70 CH_3OH:30 H_2O/90 CCl_4:10 $CHCl_3$. Figure 10 shows the approximation of the experimental points to the theoretical curve of normal distribution for pure 16α-hydroxyprogesterone. Statistical analysis of the data gave results suggesting the radiochemical purity of the material (variance ratio = 0.0173). The contents of the tubes within $\pm 2\theta$ of the peak were pooled, 10 mg. of pure 16α-hydroxyprogesterone was added and the material was crystallized from methanol (Table 3). A total of 15,800 counts was present in the material when first crystallized. The first crystals and mother liquor contained 13,400 counts (85% recovery of radioactivity). The first crystals (8,800 counts) were recrystallized from absolute methanol and crystals and mother liquor contained 8,200 counts. The second crystals (1,880 counts) were acetylated with redistilled acetic anhydride and pyridine and the acetate when recrystallized contained 1,900 counts in the crystals and mother liquor. The specific activities of the crystalline 16α-hydroxyprogesterone and its derivative were the same within experimental error (Table 3). The infra-red spectrum of the acetate derivative was identical to that of authentic 16-acetoxy-progesterone.

Similar radioactive experiments in which homogenized fetal adrenals were incubated with acetate-2-C^{14}, mevalonic-2-C^{14}, or cholesterol-4-C^{14} failed to yield any radioactive material in the regions of the 6 steroid carriers used in the progesterone experiments.

DISCUSSION

The demonstration of the production of 17-hydroxyprogesterone in the human fetal adrenal corroborates the work of Solomon and Lanman (2). The present work suggests that 17-hydroxylase is active even in the very youngest fetus tested (Figs. 3 and 4). The activity of the enzyme could be demonstrated in incubations of either tissue slices or homogenates and does not require the addition of a TPNH-generating system (Figs. 5, 6, and 7). Previous experiments with glucose-1-C^{14} and glucose-6-C^{14} have provided evidence of a very active phosphogluconate shunt in fetal adrenals (3) which could supply the required TPNH.

Deoxycorticosterone has been isolated as a product of progesterone metabolism by Plager and Samuels (7). This steroid is not found in the blood or urine of the normal human; however, it is excreted in significant quantities in the patient with the hypertensive form of adrenocortical virilism (8). In these experiments with the human fetal adrenal, deoxycorticosterone was not formed prior to age 10 weeks, it appeared in relatively large amounts in the adrenals of fetuses from age 10–15 weeks, and was present in a very small amount in the adrenal of the fetus of age 25 weeks (see Table 4). These results suggest that the enzyme, 21-hydroxylase is absent (or inactive) in the early weeks of gestation. If such were the case, pathway 1 (see Fig. 11) would predominate in the adrenal of the very young fetus. If the activity of the enzymes forming Substance S and

cortisol (21-hydroxylase and 11-hydroxylase) were diminished (as shown by the small amount of radioactivity associated with carrier cortisol, even with the addition of TPNH), pathway 3 to androstenedione would be favored. One could thus compare the very early fetal adrenal with that of the patient lacking 21-hydroxylase (usual form of adrenocortical virilism) and consequently accumulating large amounts of 17-hydroxyprogesterone and adrenal androgens (9).

In the human fetal adrenal 21-hydroxylase activity appears to reach a maximum from about 10 to 15 weeks gestation. With an increase in the activity of pathway 2, 17-hydroxylation may be diminished and this might

TABLE 4. SUMMARY OF RADIOACTIVE SUBSTANCES FORMED

Adrenal	Medium	Co-factors	C^{14} in regions of carriers					
			And.	DOC	17OH Prog.	16OH Prog.	B	F
I	$KMgPO_4$	C	++	0	++++	++++	?	+
II	$KMgPO_4$	C						
III	$NaKPO_4$	C	?	0	++	+	+	+
IV	$KMgPO_4$	C	0	0	++	++++	++++	++++
V	$NaKPO_4$	N	0	0	++	++	++	++
VI	$KMgPO_4$	N	0	++	+++	++	0	0
VII	$KMgPO_4$	N	0	++*	++*	++*	0	0
VIII	$KMgPO_4$	N	0	+*	+++*	++*	0	0

0 = No radioactivity found
\+ = Less than 30,000 cpm
++ = 30,000–100,000 cpm
+++ = 100,000–200,000 cpm
++++ = Over 200,000 cpm
C = TPN and glucose-6-phosphate added
N = No cofactors
* = Evidence for radiochemical purity obtained

account for the disappearance of androstenedione. This stage of development of the fetal adrenal might be compared with that of the patient with the hypertensive form of adrenocortical virilism in which elevated levels of deoxycorticosterone are found in the blood and urine (8). The excretion of adrenal androgens is elevated, however, in these patients.

The adrenal of the older fetus more nearly approaches the normal adult level of production of deoxycorticosterone. The decrease in amount of deoxycorticosterone formed in the older fetal adrenals may represent increased activity of pathways from deoxycorticosterone to corticosterone (11-hydroxylation), to aldosterone (18-hydroxylation) or to other, as yet unknown, products.

This grossly oversimplified picture of the complex metabolic pathways of steroid biogenesis should serve primarily to focus attention on the importance of competition of various enzymes for substrates and cofactors (such as TPNH), and to reinforce the suggestion that certain enzymes may develop at a later time than other enzymes, changing the flow of materials

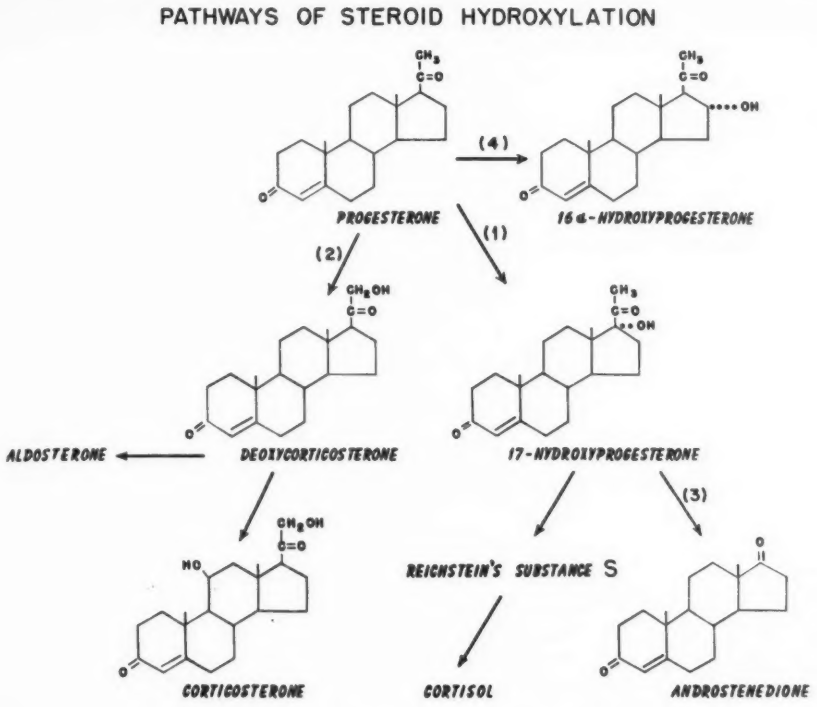

FIG. 11. Pathways of Steroid Hydroxylation.

through these paths of steroid biosynthesis and yielding quantitative and qualitative differences in the steroids produced.

Perhaps the most interesting finding is the demonstrated production of 16α-hydroxyprogesterone by the human fetal adrenal. 16-Hydroxylation of estradiol has been reported in human fetal liver (10), but 16-hydroxylation of progesterone has not been found previously in human tissue. The steroid, 16α-hydroxyprogesterone, has been found as a product of progesterone metabolism in hog adrenal homogenates (11, 12). 16-Hydroxylated progesterone derivatives were found in the urine of a patient with adrenocortical carcinoma (13) and a reduction product, 3β, 16α-dihydroxy-5α pregnane-20-one, has been isolated recently from the urine of patients with the salt-losing form of adrenocortical virilism (14). Bernstein (15) has discussed the sodium diuretic properties of many 16-hydroxylated steroids Thus the possibility exists that 16α-hydroxyprogesterone is the elusive "salt-losing hormone." Coppage and Liddle (16) reported that 3β, 16α-dihydroxy-5α pregnane-20-one had no sodium diuretic effect when administered to human subjects. Since this compound is a reduction product of 16α-hydroxyprogesterone, and therefore quite probably inactive, their results are not

surprising. If because of a genetic lack of the enzyme (or enzymes) involved in the 21-hydroxylation of 17-hydroxyprogesterone, these pathways are blocked, alternative pathways of metabolism, possibly involving 16-hydroxylation of the progesterone molecule, would become more active. If 16α-hydroxyprogesterone does possess sodium diuretic activity, its presence in increased amounts might explain some of the clinical symptoms observed in the patient with salt-losing adrenocortical virilism.

Acknowledgments

We wish to thank Professor John Lind, Department of Pediatrics, Karolinska Hospital, and Professor Carl Schmitterlow, Department of Pharmacology, Kungl. Veterinärhögskolan for their generosity in providing the fetal specimens and the laboratory facilities for the experiments in Stockholm. We would like to express our gratitude to Dr. Seymour Bernstein of the Lederle Laboratories and Dr. Harold Upjohn of the Upjohn Company for providing us with samples of authentic 16α-hydroxyprogesterone. We wish to thank Miss Norma Madeira who carried out the infra-red analyses and Mrs. Marguerite Cahoon, Miss Dana Gedmintas and Miss Celia Hillman for their expert technical assistance.

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EFFECTS OF EXOGENOUS ANDROGEN ON SEXUALLY RESPONSIVE AND UNRESPONSIVE MALE RATS[1]

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ABSTRACT

A certain proportion of any group of male rats will fail to initiate sexual behavior when tested with receptive females. These males have been termed "non-copulators." In the present study the sexual responsiveness of castrated "copulator" and "non-copulator" male rats to increasing levels of exogenous testosterone propionate was tested. Hormone doses ranged from 0 μg to 200 μg/100 gm. body weight. All "copulator" males copulated and ejaculated before or at the 90 μg androgen level. Only one of the six "non-copulator" males initiated mating during the experiment, and none ejaculated. These data were interpreted to mean that "copulator" and "non-copulator" male rats differ in their ability to make use of circulating androgen. Additional data indicated that sexual responsiveness is directly related to androgen level, and that the ejaculatory process is minimally related to the hormone dose.

EXPERIMENTERS investigating the sexual behavior of laboratory rats have found that every colony includes a certain number of apparently healthy males which fail to copulate when placed with receptive females in a standard testing situation (1, 2, 3, 4, 6, 7, 13, 15, 16, 17, 18). This phenomenon can be illustrated by combining the results of several different experiments conducted in the psychological laboratories at Yale University and at the University of California.

A total of 129 male rats were observed in a series of 10-minute tests with receptive females. The cumulative percentage of animals acheiving their initial intromission in each test was as follows: 54.3, 68.2, 75.2, 82.9 and 86.0 Experience has shown that further testing might result in the exhibition of copulation by a few more males, but that the cumulative curve of responding animals reaches asymptote well below the 100% mark.

Received January 17, 1961.

[1] The research, which was conducted at the University of California, Berkeley, was supported by a research grant from the USPHS (M-943) to F. A. Beach. Hormones were supplied by Dr. Edward Henderson, Schering Corporation, Bloomfield, New Jersey.

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The percentages presented above show that quite a few males which fail to copulate in the first test succeed in doing so when retested several days later, and, it should be added, once mating has begun it continues to occur on subsequent occasions. Failure to respond sexually in initial tests seems, in some cases, to be due to distraction or fear generated by the novelty of the testing situation. Repeated exposure to this situation results in habituation to its nonsexual aspects and permits the male to react to sexual stimuli provided by the estrous female.

There are at least four possible explanations for the persistent lack of sexual responsiveness in some individuals beyond the initial tests.

(1) It could be postulated that males which never copulate are individuals that never become sufficiently adapted to the test situation. Opposed to this interpretation is the fact that during later tests such animals do not exhibit the signs of "nervousness" or "fear" which frequently characterize the behavior of males that fail to mate in early tests but later succeed in doing so.

(2) A second possibility is that sexually unresponsive males are deficient in the secretion of testicular androgen; but the histological normality of the accessory reproductive glands of these individuals argues against this explanation.

(3) Thirdly it might be supposed that although they secrete normal amounts of androgen, persistent non-copulators are much less sensitive to the hormone than are sexually responsive males. It is conceivable that central nervous mechanisms which are normally influenced by androgen are refractory in the non-copulating male. This hypothesis is supported by the finding that some, though not all, "non-copulating" male rats may begin to mate after being injected with relatively massive doses of testosterone propionate (5, 9, 17).

(4) Finally, it may be that the neural or neuromuscular mechanisms essential for mating are absent or incompletely organized in those males that fail to copulate.

The present experiment was designed to test systematically the second and third of these explanations. The plan was to select copulating and non-copulating males, castrate them, and then test their reactions to receptive females under the influence of exogenous androgen. If the original lack of sexual responsiveness was due to a deficiency of endogenous androgen, then the amount of testosterone propionate which would produce copulation when injected into castrated copulators should suffice to evoke the same response in former non-copulators. On the other hand, if failure to copulate before castration was due to relative insensitivity to endogenous testosterone, it would be expected that larger amounts of injected hormone would be needed to evoke postoperative mating in former non-copulators than in preoperative copulators.

METHODS

Subjects

The 23 Long-Evans rats used in this study were selected from a group of 30 males on the basis of performance in preliminary mating tests. All males were approximately 180 days old at the time of the initial mating test and weighed 433 gm. on the average Subjects were maintained on a reversed light-dark cycle and *ad libitum* feeding.

Selection Tests

The males were divided into "copulator" and "non-copulator" groups according to performance with estrous females in the course of five 15 minute mating tests. In these tests, which were conducted at one-day intervals, males were allowed no more than two intromissions and no ejaculations. If a male achieved intromission during any one of the five tests, he was classified as a "copulator." Males which failed to achieve intromission during at least one of the five tests were classified as "non-copulators." Eighteen of the 30 males (60%) copulated. A random sample of 12 males was selected from these 18 for experimentation. The "non-copulator" group consisted of 12 males which did not respond sexually during the selection tests.

Castration and Androgen Replacement

Within one week after the selection tests all 12 "copulators" and six "non-copulators" were castrated. The remaining 6 "non-copulators," one of which died during the experiment, were kept intact as control subjects for group NC.

Beginning two weeks after gonadectomy, testosterone propionate in oil was administered to all castrates by subcutaneous injection. On days 1–6 hormone was administered within approximately one hour after the start of the 12-hour dark phase of the 24-hour cycle. Approximately five hours after the injection on day 6, each *S* was given an opportunity to mate with an estrous female. Non-castrate, non-injected "non-copulators" were tested at the same time. On day 7 no hormone was administered, and treatment was resumed on day 8. The seven day cycle (i.e. 6 days of injections followed by one of no treatment) was repeated with a steadily increasing dose level until each male had ejaculated on three consecutive weeks, or until it was judged unlikely that mating would occur. The doses of testosterone propionate in μg/100 gm. body weight, were as follows: 0, 2, 10, 15, 20, 25, 30, 35, 45, 60, 70, 80, 90, 100, 110, and 200.

Mating Tests

All tests were conducted during the dark phase of the 24-hour cycle. The semi-circular observation cages in which mating occurred have been described previously, as has been the procedure for bringing stimulus females into weekly estrus (12).

Three minutes before the start of a test a male was placed in the observation cage. At the end of the adaptation period a receptive female was introduced and mating was allowed to occur until one ejaculation had been achieved, or until 15 minutes had passed without the occurrence of an intromission.

The occurrence and timing of mating responses were recorded by *E* on a constant speed kymograph. The behavioral measures were as follows: *Mount latency:* time from the introduction of the female until the male's first mount without intromission; *Intromission latency:* time from the introduction of the female until the male's first intromission; *Intromission frequency:* number of intromissions before ejaculation.

RESULTS

In the course of the nine weekly mating tests administered to the control *S*s, 4 of the 5 intact "non-copulator" males initiated sexual behavior. Two

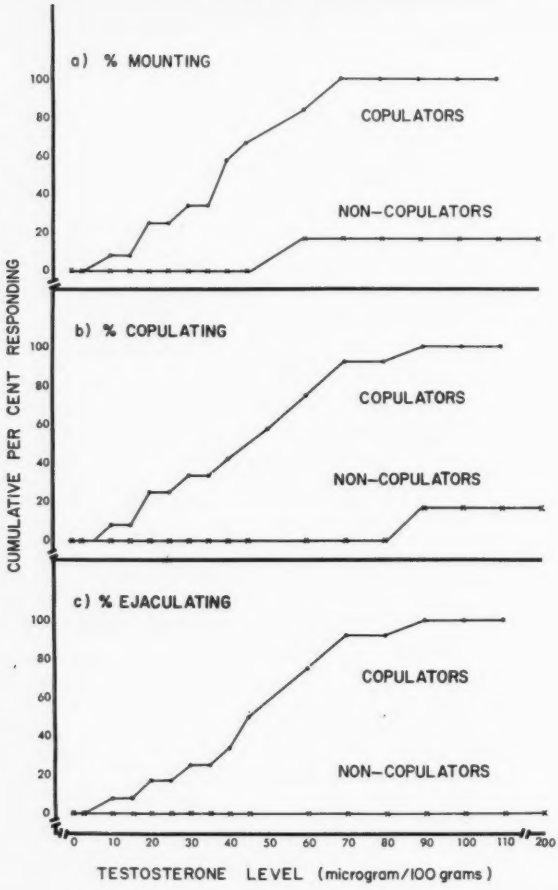

FIG. 1. Cumulative percentages of two groups first showing three different sexual responses at different levels of androgen treatment.

males copulated for the first time during the first control test, one copulated on the second, and the fourth male achieved intromission during test 3.

Comparison of the castrated "copulators" and "non-copulators" reveals that the two groups responded differentially to exogenous testosterone propionate. This is shown in Figure 1 which represents the cumulative percentages of males achieving their initial (a) mount without intromission, (b) intromission and (c) ejaculation, as a function of dose level. For each measure there was a progressive increase in the proportion of the "copulator" group which responded. The curves indicate that mounting without intromission was the response most sensitive to the increase in hormone

level. This is shown by the fact that the curve representing percent of group mounting (Figure 1a) reached 100% at a lower dose level than did the other two curves. The curves for the percent copulating and percent ejaculating were nearly coincident over their course, indicating that if a male achieved intromission in a particular test he was likely to continue copulating until ejaculation occurred.

Five of the six "non-copulators" failed to exhibit sexual behavior in response to testosterone propionate, even though the dose level was brought up to 200 μg/100 gm. body weight. The one male which did respond mounted the female for the first time at a hormone level of 60 μg and achieved intromission at the 90 μg level, but never ejaculated.

DISCUSSION

Results of this experiment raise several pressing questions. (1) Why did four of the five unoperated "non-copulators" eventually begin copulating? The most likely explanation seems to be that these were animals which were relatively slow in becoming habituated to the observation cage and the testing procedure. Furthermore it is obvious that individuals differ in the ease with which they become sexually aroused, and it seems probable, as suggested by Beach (8) that males exhibiting different degrees of susceptibitlity to sexual arousal can be assigned different positions on a unidimensional scale.

> Relatively inexcitable individuals fall along the lower portions of this scale. At the opposite extreme . . . belong those males in which sexual excitability is quite high. Between these two end points are found the majority of inexperienced males displaying average degrees of excitability. Although present knowledge permits only the assumption that such variation does exist, it seems likely that careful investigation of a large population of inexperienced males would reveal a normal distribution of excitability, with a mean falling near the center of the . . . scale. (p. 176)

According to this point of view, most of the "non-copulators" in the present experiment might be thought of as belonging perhaps 1.5 to 2.0 standard deviations below the mean on a scale of sexual excitability. Males failing to copulate under any circumstance would belong even lower on the scale. That such males exist is suggested by the fact that some intact "non-copulators" fail to mate despite the administration of exceedingly large amounts of testosterone propionate (5, 9, 17).

(2) Since four of the five intact "non-copulators" began to copulate after repeated testing, why did five of six castrates fail to do so? Unless this difference is attributed entirely to chance, it must be referred to some effect of the operation and subsequent hormone treatment. It is to be expected that castration would drastically reduce sexual excitability, but the amounts of androgen administered during the latter stages of the experiment were more than enough to compensate for loss of the gonads, and certainly exceeded greatly the normal output of testosterone. It is

possible that the procedure of starting with a very low dose and gradually increasing it had an inhibiting effect upon the response to the higher levels reached toward the end of the experiment.

(3) One castrated "non-copulator" eventually began to copulate under the influence of testosterone propionate injections, but only after the administration of much more hormone than was needed to restore sexual behavior in the "copulator" group. Why was this the case, and why did the "non-copulator" fail to ejaculate? The finding that more androgen was needed to stimulate sexual activity in the "non-copulator" is in accord with the hypothesis that males of this type are less responsive to the hormone than are "copulators." The absence of ejaculation suggests that the threshold for this response is higher than the threshold for copulation.

The evidence points to the conclusion that "copulator" and "non-copulator" males differ in their ability to make use of exogenous androgen. Such a difference could reflect differential sensitivity of critical "target tissues," or it could result from a difference in the rate at which the hormone is metabolized or deactivated. Present findings provide no basis for choosing between these two alternatives. The results of this experiment are contrary to the hypothesis that the difference between copulators and non-copulators is due solely to differences in the production of testosterone. This is in harmony with the conclusion of Beach and Fowler (10) to the effect that individual differences in the sexual performance of male rats are due to a combination of factors, of which testosterone level is probably one.

In addition to the planned comparisons between the experimental groups which have been discussed, certain changes over time in the performance of the "copulator" group are worth noting. In this experiment 100% of the "copulators" exhibited ejaculation only after the hormone level had reached 90 μg/100 gm. body weight (Figure 1c). Since at that point in testing, the average body weight for the group was approximately 500 gm., each "copulator" male was receiving about 450 μg of testosterone propionate on each of the six days preceding the mating test. This dosage is 6–9 times larger than the dose Beach and Holz-Tucker (11) found necessary to maintain castrated rats at pre-operative levels of sexual performance. These workers began hormone administration shortly after gonadectomy, and used dosages considerably in excess of the beginning dose in the present study. It is possible that allowing three weeks for the dissipation of endogenous hormone and then slowly increasing the amount of amount of androgen administered reduces the effectiveness of larger doses when they are eventually employed. Alternatively, the different results of the two experiments may be due to differences in the strains of rats used as subjects.

Even though the "copulator" males did not mate at as low a hormone level as previous reports would indicate to be adequate, present results do replicate earlier findings in some respects. As is shown in Table 1, mount

TABLE 1. CHANGES IN SEXUAL BEHAVIOR SCORES AS RELATED TO INCREASES IN TESTOSTERONE PROPIONATE LEVEL

Measure	First relevant test	Third ejaculation test	Proportion of group changing	p
Median Mount Latency (sec.)	72.0	9.0	11/12 decrease	$< .001$
Median Intromission Latency (sec.)	160.0	17.0	10/12 decrease	$< .02$
Mean Intromissions Before Ejaculation	7.2	8.3	7/12 increase	not signif.

First relevant test refers to the first test on which the measure, e.g., mount, occurred for the first time. Probability values were determined by the sign test.

and intromission latencies decreased from the first test on which mounting or intromission occurred to the third test on which ejaculation occurred. That is, as testosterone propionate level increased, latencies decreased.[3] These data parallel the findings of Beach and Holz-Tucker (11) who noted that for groups receiving different amounts of androgen there was a marked inverse correlation between dose level and response latency. This is also related to the finding of Beach and Fowler (10) who reported an inverse relation between dose and latency for different individuals receiving different amounts of hormone.

The only finding not congruent with earlier reports concerns the frequency of intromissions preceding ejaculation. Beach and Holz-Tucker (11) stated that when the androgen dose was increased from approximately 9 μg/100 gm. to 25 μg/100 gm. intromission frequency before ejaculation increased from 9.3 to 11.1 ($p < .01$). Present data showed no statistically significant increase in intromission frequency when the average increase in testosterone level was 19.2 μg/100 gm. (Table 1). In fact, 5 of the 12 males in the group showed either no change or a decrease in intromission frequency as dose increased. In addition, there was no correlation between intromission frequency before ejaculation and the dose level at which ejaculation first occurred. That is, males which ejaculated for the first time at the 90–100 μg androgen level did not achieve more intromissions before ejaculation occurred than did males which ejaculated for the first time at the 20–30 μg level.

The data in Table 1 therefore support the conclusion that sexual responsiveness, as measured by mount or intromission latency, is directly related to the level of circulating testosterone propionate. In contrast, although a certain concentration of androgen is essential to the occurrence of ejaculation, the administration of additional amounts of hormone has no effect upon the ejaculatory process.

[3] It should be noted that in these comparisons the increase in androgen level is confounded with the number of mating tests given. Changes in latency do occur with repeated tests for intact males but the changes are not of the magnitude found here (19)

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DISTRIBUTION OF GONADOTROPHIC AND ADRENOCORTICOTROPHIC ACTIVITY IN THE ANTERIOR PITUITARY GLAND OF THE MALE RAT[1]

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ABSTRACT

Gonadotrophic and ACTH activity were assayed in a segment of the pituitary rich in peripheral gonadotrophs (delta cells) and in an area almost devoid of this type of cell. The region rich in delta cells was found to contain three times the gonadotrophic activity and one-third the ACTH activity of the region poor in delta cells. Thus the delta cells are the site of gonadotrophin and not of ACTH production.

THE cellular site of gonadotrophin and thyrotrophin production in the anterior pituitary of the male rat is now fairly well established mainly through a series of observations by Purves and Griesbach (1, 2, 3, 4, 5, 6). Three types of basophils or PAS positive cells are recognized. FSH is thought to be produced by the oval, PAS positive, aldehyde-fuchsin negative peripheral gonadotrophs (delta cells) which are concentrated in the two lateral ridges on the supero-anterior surface of the anterior pituitary ("sex zone") (1, 4, 5, 7, 8, 9). The LH (ICSH) producing PAS positive, aldehyde-fuchsin negative central gonadotrophs are thought to be more widely dispersed (4, 5, 7, 8, 9). The PAS positive, aldehyde-fuchsin positive, angular TSH producing cells (thyrotrophs or beta cells) are located centrally (1, 2, 3, 6, 8, 9, 10).

A large body of investigation on both animal and human pituitaries points to the basophils as the site of ACTH production as well (11, 12, 13, 14, 15, 16). In order to further investigate the problem of ACTH localization, the gonadotrophin and ACTH content of the "sex zone" has been assayed against an area almost devoid of delta cells.

MATERIALS AND METHODS

Distribution of Peripheral Gonadotrophs:

Horizontal serial sections were prepared from several male rat pituitaries and stained by the PAS technique (17). From these serial sections, using a camera lucida, paraffin reconstructions were made. The distribution of the delta cells as well as the areas almost devoid of delta cells were outlined on these reconstructions. A model of the pituitary was

Received January 31, 1961.

[1] This work was supported in part by a grant, Consolidated Grant No. 2, from the National Research Council of Canada.

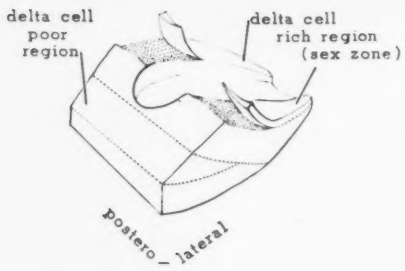

FIG. 1. Adult male rat pituitary with posterior pituitary removed, viewed from above and laterally. The areas used for bioassay are demonstrated.

thus constructed giving a three-dimensional representation of the distribution of the delta cells, allowing an exact sampling of zones in fresh pituitaries.

Preparation of Pituitary Extracts:

Pituitaries were obtained from male rats of the Wistar strain weighing 200–240 gm., kept in the animal room and handled twice or three times daily for at least one week prior to assay. The rats were injected intraperitoneally with Nembutal, returned to their cages until completely anaesthetized and then transported to the laboratory. While under general anaesthesia, they were decapitated with a guillotine. The top of the skull and brain were removed, the superior capsule of the pituitary dissected free with a No. 19 $1\frac{1}{2}''$ needle, and the posterior pituitary carefully detached and separated with the same needle. The base of the skull was then cut away. This bony plate (with the pituitary still *in situ*) was then transferred to the stage of a freezing microtome and frozen with CO_2. The time interval between decapitation and freezing was 1–$1\frac{1}{2}$ minutes. The "sex zone" segment was obtained by a free hand cut using a razor blade along each supero-anterior lateral ridge (see Fig. 1). An underlying intermediary zone was discarded. A segment of the region poor in delta cells was then obtained, leaving pituitary tissue inferiorly still attached to bone. The segments were transferred before thawing, using a fine brush, to pre-weighed squares of parafilm 5×5 mm., weighed with a microtorsion balance to the nearest 0.05 mg. and quickly transferred on the parafilm squares to the extraction tubes. Samples from at least ten anterior pituitaries were pooled for each assay. Each "sex-zone" fraction weighed approximately 0.5 mg., each delta cell-poor fraction, approximately 1.5 mg.

Tissue for use in the gonadotrophin assay was transferred to 15 cc. conical centrifuge tubes containing 1 cc. normal saline and a few grains of sand. The samples were then ground with a glass rod rounded at one end, the rod then washed with 0.5 cc. normal saline. The tubes were allowed to stand at room temperature for one-half hour, then stored in a deep freeze for 2–3 days. On the day of assay, the samples were thawed at room temperature, adjusted to a pre-determined volume with normal saline, and allowed to stand for two hours. The supernatant was then removed with a long-nosed pipette, ready for assay. A similar procedure was followed in preparing the extracts for the ACTH assay, except that the pituitaries were transferred to 0.1N. HCl and adjusted to 0.01N. HCl on the day of assay.

Volumes were adjusted so that equal volumes would be used for injection from both zones.

Gonadotrophin Assay:

A modification of Klinefelter's (18) assay was used in which the increase in uterine weight of immature female mice is the criterion for gonadotrophic potency. Intact immature female mice of the C-F #1 strain, weighing 10–12 gm. were used. After a series

TABLE 1. GONADOTROPHIN ASSAY OF ANTERIOR PITUITARY TISSUE

	Anterior pituitary (mg. fresh tissue)					
	Zone rich in delta cells			Zone poor in delta cells		
	0.020	0.033	0.060	0.060	0.100	0.180
Uterine weight (mg.)	11.3	39.6	65.2	15.2	38.3	58.3
	14.9	34.0	58.3	18.0	41.6	64.7
	13.0	40.2	68.4	17.1	39.0	66.2
	13.1	42.7	52.7	16.0	40.2	61.5
	b = 100.41			b = 95.76		

$$\text{Potency ratio: } \frac{\text{Delta Cell Rich}}{\text{Delta Cell Poor}} = 2.87$$

$S_E = 3.68$
$\lambda = 0.0375$
95% confidence interval: 2.67–3.09

of preliminary assays, a three point dose level for each extract was chosen which gave (1) a linear log dose-response curve, (2) similar quantitative responses for both zones, and (3) a response to the lowest concentration at least two times that of the control uterine weight. The extracts were injected subcutaneously twice daily for three days and the animals sacrified on the morning of the fourth day, 16 to 18 hours after the last injection. The mice were decapitated, uteri removed, blotted and weighed.

ACTH Assay:

The test animals were male rats of the Wistar strain weighing 200–220 gm., kept in the animal room and handled frequently for at least one week prior to assay. The ascorbic acid depletion assay of Sayers, Sayers and Woodbury (19) was used, modified in that extracts were injected into the dorsal vein of the penis, and hypophysectomized rats were replaced by test animals pre-treated four hours prior to left hemiadrenalectomy with a hydrocortisone[2] suspension (20). This suspension was given subcutaneously, 7.5 mg./100 gm. B.W. It was found that the test animals 1) showed no ascorbic acid depletion due to stress of operation, adrenalectomy or ether anaesthesia and 2) responded to graded doses of pituitary extract with a straight line response between log dose and ascorbic acid depletion of the same order of magnitude as in hypophysectomized rats. Adrenal ascorbic acid was measured by the method of Roe and Kuether (21), modified in that the extract was incubated at 57° C for 45 minutes.

RESULTS

A number of gonadotrophin and ACTH assays were performed. Separate extracts from ten or more pituitaries were prepared for each assay. All assays produced similar potency ratios. Tables 1 and 2 outline in detail a set of typical assay results. Standard statistical methods were used.

DISCUSSION

The gonadotrophin assay of the "sex zone" against an area almost devoid of delta cells in the adult male anterior pituitary was carried out to provide direct evidence that the delta cells do indeed produce gonado-

[2] Hydrocortisone was kindly supplied by Dr. L. L. Coleman of the Upjohn Co., Kalamazoo, Mich., U.S.A.

TABLE 2. ACTH ASSAY OF ANTERIOR PITUITARY TISSUE

Anterior pituitary (mg. fresh tissue)						
	Zone rich in delta cells			Zone poor in delta cells		
	1.99	1.00	0.50	0.40	0.20	0.10
Ascorbic acid depletion (mg. per 100 gm. adrenal tissue)	199 210 228	136 123 113	80 72 101	147 149 175	78 106 123	43 46 50
	b = 212.6			b = 183.8		

$$\text{Potency Ratio: } \frac{\text{Delta Cell Poor}}{\text{Delta Cell Rich}} = 3.16$$

$S_E = 14.97$
$\lambda = 0.0755$
95% confidence interval: 2.61–3.91

The "sex zone" contains three times the gonadotrophin activity, and one-third the ACTH activity of the segment almost devoid of delta cells.

trophin. The activity of the "sex zone" was found to be three times that of the delta cell-poor region per unit weight of fresh pituitary tissue. Since the assay used will respond both to FSH and LH, and since the LH producing central gonadotrophs are thought to have a more diffuse distribution, a potency ratio of three to one points firmly to the delta cells as gonadotrophin producers. The role of the peripheral and central gonadotrophs in FSH and/or LH production would be more firmly established by specific FSH and LH assays of the "sex zone" against a delta cell-poor area.

Rochefort and Saffran (22) assayed horizontal, sagittal and coronal serial sections of adult male rat pituitaries for ACTH and found no significant differences in ACTH activity in the different sections. However, their sections were not related to specific cell groupings. Our assay procedure shows the ACTH activity of the "sex zone" to be one-third of that in the delta cell-poor region. This rules out the delta cells as the major site of ACTH production.

Acknowledgments

The authors are grateful to Professor Charles H. Best for his invaluable encouragement and advice. They wish to thank Mr. T. M. Gardner for technical assistance and Miss M. T. Wishart of the Medical Art Department for the drawing of the pituitary.

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NOTES AND COMMENTS

EFFECTS OF VASOPRESSIN ON SODIUM, POTASSIUM AND WATER DISTRIBUTION IN RAT GASTROCNEMIUS MUSCLE[1]

ABSTRACT

Muscle and plasma analyses 1, 3, 5, 8, or 18 minutes after vasopressin injection (25 mU./100 gm.) in separate groups of rats showed an immediate intracellular gain of water and sodium with a net rise in $[Na]_i$ and fall in $[Na]_o$ and converse changes in K. A rebound effect was noted at 18 minutes. Na, K and water moved at separate rates.

It has been maintained that in the mammal, the neurohypophysis helps to regulate salt and water balance exclusively through its effects on the kidney. In this the mammal would stand apart from other vertebrates. We have suggested that this view cannot be entirely correct since vasopressin, like any other vasoconstrictor, causes a marked decline in extracellular Na and some rise in K in the nephrectomized rat or dog (1, 2). These changes represent a true shift of cation from one compartment to another for, in studies of the aorta wall, it was noted that vasopressin caused an intracellular gain in Na and loss in K at the time of peak rise in blood pressure (3). Ingle and Li observed that small amounts of vasopressin act to normalize the work performance of the adrenalectomized-hypophysectomized rat (4). Since the ability to perform work also involves an ability to effect transmembrane ionic exchanges (5) it seemed to us that the extrarenal effect of vasopressin might well apply to skeletal as well as vascular muscle. The present experiments show that the effect of vasopressin on cationic distribution can be demonstrated in the normal, non-stimulated rat gastrocnemius.

METHODS

Adult male albino rats of an inbred Wistar strain weighing 290–310 gm. were used throughout. Since preliminary experiments showed that handling, feeding, and anesthesia are important variables, special care was taken to secure uniform handling, to withdraw food on the previous evening, and to apply deep anesthesia in each case.

The animals were anesthetized in the morning with intraperitoneal pentobarbital (3.33 mg./100 gm.) and subcutaneous phenylbarbitone (6 mg./100 gm.) sodium. The kidneys were then decapsulated and the pedicles ligated. An inulin solution, approximately 25% w/v., was injected into the tail vein in a dose of 0.5 ml./100 gm. and the absolute amount of inulin injected was accurately determined in each case by careful analysis of an aliquot taken from the injection syringe. After 3 hours of equilibration, polyethylene catheters were implanted into the right femoral artery and vein and an initial blood sample (0.9 ml.) taken through the cannulated artery. A corresponding initial muscle sample was immediately excised from the right gastrocnemius and the wound sutured. Vasopressin (Pitressin—Parke, Davis) in a dose of 80 mU. (in 0.2 ml. saline), approximately 25 mU./100 gm., or the saline alone in control groups was at once administered through the femoral vein. A second muscle (left gastrocnemius) and blood sample were taken after either 1, 3, 5, 8, or 18 minutes. Separate control (saline) and test (vasopressin) groups were used for each interval making a total of 10 groups with 6 or more animals in each control group, 8 or more in each test group. The conventional analytical procedures, which in general follow Ledingham (6), have been fully presented elsewhere (5). The volume distribution of inulin in plasma was taken as a measure of extracellular fluid volume (e.c.f.v.) and, in the excised muscle sample, as a measure of muscle extracellular fluid volume (e.c.f.m.). Intracellular water was calculated by subtracting e.c.f.m. from the measured total water content of the whole muscle (per kg. fresh fat-free muscle). Intracellular Na and K were calculated by deducting from the total amount of tissue ions the amount contained in a known volume of extracellular water.

Received November 18, 1960.

[1] This work was carried out with the aid of a grant from the Muscular Dystrophy Association of Canada.

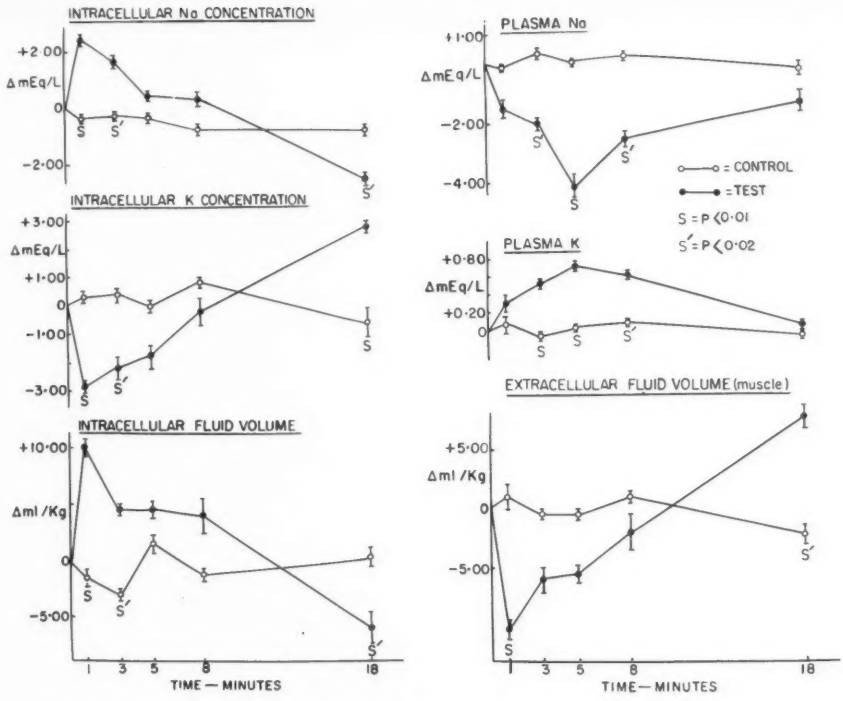

FIG. 1. Changes in the distribution of Na, K and water in rat gastrocnemius following the intravenous injection of vasopressin (approximately 25 MU./100 gm.). The effects were measured in separate groups for each interval. Vertical lines represent standard error.

RESULTS AND DISCUSSION

The findings for the 10 groups studied are presented in simplified graphic form in Figure 1. The results are shown as change from basal values measured over each interval. In brief, vasopressin produces clear-cut movements of water and sodium into and potassium out of the intracellular compartment. The rates of movement are separable. The initial cationic shift tends to reverse in a clear rebound 18 minutes after injection. (Similar exchanges can also be demonstrated in the absence of the adrenals.)

These findings bear on two basic issues: first, the question of whether exogenously administered vasopressin acts extrarenally on salt and water distribution and second, whether the neurohypophysis itself exerts similar actions. The experiment presented here leaves little room for doubt on the first issue. Vasopressin shifts Na and water out of the extracellular space into skeletal muscle. This corresponds to the effect previously shown for vascular muscle (1, 3). Ingle and Li have identified vasopressin as a factor directly involved in the performance of muscular work in the adrenalectomized-hypophysectomized rat (4). This property probably reflects the ability of vasopressin to regulate ionic exchanges (5). In fact, there is good reason to suggest, from our current studies with the sodium electrode (7), that the fact that vasopressin is vasoconstrictor is itself good evidence that it must act directly on transmembrane ionic movements.

The second problem hinges on the matter of dose, for it may be urged that since an antidiuretic effect can be elicited with a thousandfold smaller dose, the extrarenal effect described here must be entirely unphysiologic. We suggest that this position is untenable, however, since the rat with diabetes insipidus induced by neurohypophyseal denervation shows, in fact, a marked expansion of the extracellular space coupled with a small but definite increase in plasma Na, changes which occur even in the absence of the kidneys (8). Experiments in progress indicate that the replacement dose for such diabetic rats is considerably larger than the dose used here.

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THE *IN VITRO* METABOLISM OF PROGESTERONE-C^{14} TO $\Delta^{1,4}$-ANDROSTADIENE-3,17-DIONE BY A CYSTIC BOVINE OVARY[1,2]

ABSTRACT

A metabolite of the *in vitro* metabolism of progesterone-C^{14} by a cystic bovine ovary was identified as $\Delta^{1,4}$-androstadiene-3,17-dione. Of the total amount of progesterone-C^{14} added, 0.0142% was converted to the androgenic substance.

The study of progesterone-C^{14} metabolism by bovine ovarian tissues was initiated to investigate the *in vitro* metabolic pathway of progesterone-C^{14} by the homogenates of normal and cystic bovine ovaries.

In 1954 Hayano *et al.* (1) reported the *in vitro* conversion of progesterone to Δ^4-pregnene-20β-ol-3-one by bovine corpus luteum tissues. Later (1956) Solomon (2) demonstrated the *in vitro* formation of 17α-hydroxyprogesterone and Δ^4-androstene-3,17-dione from progesterone by bovine ovarian tissues, thus indicating that progesterone may be a precursor in the biogenesis of androgens.

This report includes a preliminary study of the *in vitro* metabolism of progesterone-

Received November 25, 1960.

[1] Supported in part by grant S-59-6 from the National Academy of Sciences-National Research Council, Division of Medical Sciences.

[2] Contribution No. 614, Department of Chemistry, and No. 292, Department of Dairy Husbandry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, Kansas.

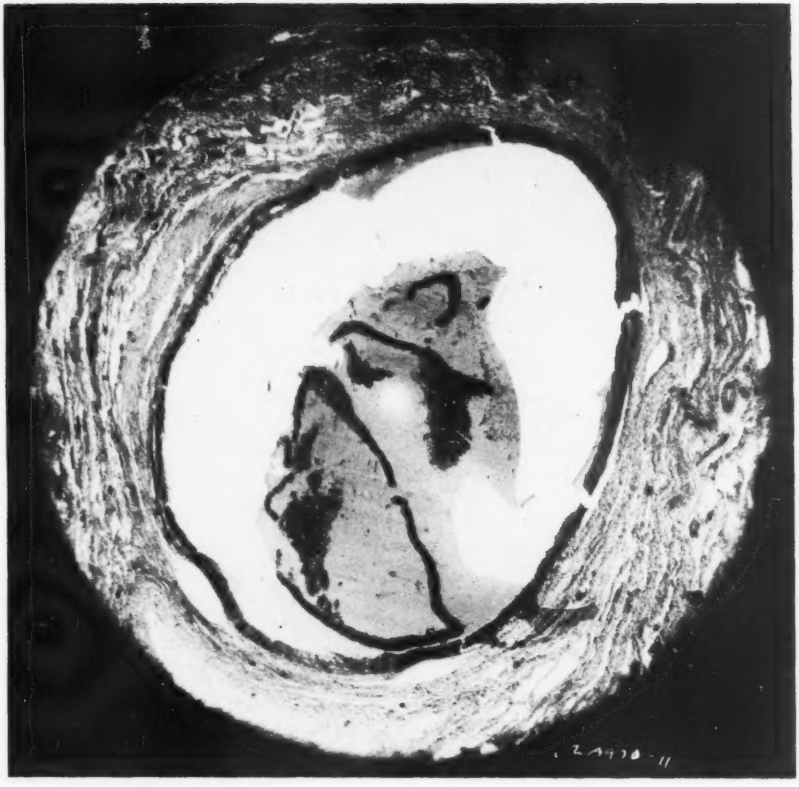

FIG. 1. Single follicle cyst. Hematoxylin and eosin. ×100.

4-C^{14} to $\Delta^{1,4}$-androstadiene-3,17-dione by a cystic bovine ovary. The bovine ovary (88.9 gm.) contained a large, single, non-luteinized cyst with 17 cc. of follicular fluid (Fig. 1). It was obtained at slaughter from a cross-bred diary cow which had an elevated tail head and relaxed pelvic ligaments.

The incubation medium was essentially that used by Samuels (3). Two grams of the frozen bovine ovary were sliced from the center and homogenized with a serum-buffer mixture. The medium included an equal volume of bovine serum and phosphate buffer at pH 7.4 containing the following concentrations of co-factors: 0.04 mole nicotinamide, 0.35 millimole diphosphopyridine nucleotide, 0.31 millimole adenosine triphosphate, and 0.4 millimole furmaric acid. The homogenate was transferred into a 125 cc. glass stoppered Erlenmeyer flask and diluted with the serum-buffer mixture to a 25 cc. volume for the incubation.

One micromole of progesterone-4-C^{14} was then dissolved in 0.2 cc. of propylene glycol and added to the incubation flask. Incubation was carried out under a gas phase of 95 percent oxygen and 5 percent carbon dioxide for three hours with constant shaking in a water bath at 37° C.

The lipid fraction of the incubation mixture was extracted four times (v/v) by ethyl

acetate, from the serum-buffer mixture. Some lipids were then removed by placing the extract on a column (110×11 mm.) containing 4 gm. of silica gel. For further separation, the partially defatted extract was chromatographed on Whatman No. 1 filter paper using Savard's method (4).

To detect and identify the steroids, the extract was subjected to paper chromatography by two solvent systems. First, a toluene-propylene glycol system was used to separate radioactive compounds which were detected by a paper chromatogram scanner. Also a ligroine-propylene glycol system revealed one radioactive spot which was eluted from the paper and rechromatographed three times.

The unknown compound had an R_T value of 0.67, which was similar to the 0.62 R_T (4) of $\Delta^{1,4}$-androstadiene-3,17-dione in the ligroine-propylene glycol system. The radioactive spot moved with the standard $\Delta^{1,4}$-androstadiene-3,17-dione located on an adjacent paper strip.

Before the radioactive compound was rechromatographed a second time, it was mixed with 150 μg of $\Delta^{1,4}$-androstadiene-3,17-dione as a carrier. The unknown and the standard hormone had the same mobility on the chromatogram which was verified as a single dark spot by scanning the chromatogram with ultraviolet light. A pronounced radioactive peak was also located in the area of the dark spot by a paper chromatogram scanner. Since there was only one spot, the compounds were presumed to be identical. Both the unknown and the standard had the same mobility in a toluene-propylene glycol system.

Additional identification of the unknown was made by infrared spectrophotometric analysis. The characteristic absorption peaks of the spectrum were found to be identical with those of the spectrum of standard $\Delta^{1,4}$-androstadiene-3,17-dione. The micro quantity was analyzed by using a potassium bromide pellet (1½ mm.) and a beam condensing unit (Perkins-Elmer) with an Infracord spectrophotometer.

The radioactive substance and the carrier were eluted with ethanol and counted in a gas flow proportional counter. A total activity of this compound indicated a 0.0142 percent conversion from 1 micromole of the incubated progesterone-4-C^{14}.

On three other trials with normal bovine ovaries, *in vitro* conversion of progesterone-C^{14} to $\Delta^{1,4}$-androstadiene-3,17-dione was not detected. Also a similar experiment with a polycystic bovine ovary did not reveal the androgenic substance.

In 1956 Miller *et al.* (5) isolated $\Delta^{1,4}$-androstadiene-3,17-dione in the feces of a pregnant cow injected with 5 grams of progesterone. They suggested that progesterone was first metabolized to Δ^4-androstene-3,17-dione and then to $\Delta^{1,4}$-androstadiene-3,17-dione. Miller *et al.* also believed it pertinent to draw attention to the relatively large amount of endogenous and exogenous progesterone in the animal at the time of conversion. Their findings may aid in explaining why $\Delta^{1,4}$-androstadiene-3,17-dione was detected in the cystic ovary, since the follicular fluid (17 cc.) of the ovarian cyst contained 143 μg of progesterone, as determined by the method of Gawienowski and Mayer (6).

The role of $\Delta^{1,4}$-androstadiene-3,17-dione in the physiology of reproduction should be more thoroughly investigated. Isolation of this hormone from a cystic ovary but not from normal bovine ovaries may indicate a difference in the pathway for progesterone in the infertile animal.

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OXYTOCIC ACTIVITY OF HUMAN FEMALE BLOOD

ABSTRACT

Using the Bristol Procedure for the extraction of oxytocin from blood taken from healthy non-pregnant adult females and from women during normal pregnancy, we have found both oxytocin and oxytocic substance (O.S.) to be present in the extracts. Precautions were taken to remove the possibility that potassium, 5-hydroxytryptamine, acetyl choline and plasma kinin in the extracts might be interfering substances in the assay procedures. The extracts were assayed on the isolated sensitised rat uterus using both the superfusion and classic methods. The results obtained confirm our previous observations that there is more oxytocin in the blood during pregnancy than in non-pregnancy. We also found more O.S. in the former than in the latter conditions.

We have previously reported finding oxytocin and oxytocic substance (O.S.) in extracts of blood from men, women, sheep, cows, goats and rats (1, 2, 3, 4, 5, 6, 7, 8, 9). The method used for the extraction of oxytocin and oxytocic substance from blood was that described by Bisset and Walker (10). This method entails acid-alcohol extraction of the blood, and since acidification is known to liberate oxytocic polypeptides from the plasma proteins (11), we decided to use an entirely different method of extraction.

Accordingly, we have employed the Bristol method (12) for extracting oxytocin from blood, and with this method we have determined the oxytocic activity (O.A.) of blood taken from non-pregnant females and from healthy females during normal pregnancy.

The results obtained with this new method confirm our previous observations that oxytocin is present in higher concentration in blood during pregnancy than in the non-pregnant state (1).

METHOD

Preparation of the Extracts

The Bristol Procedure of extraction was used (12) and this is briefly described as follows: Samples of venous blood were collected from the ante-cubital veins of the various subjects and prevented from clotting by adding 100 I.U. of heparin in 1.0 ml. of isotonic saline to every 10 ml. sample of blood. The blood was taken through a siliconed needle into an ice-cold polythene syringe and then put into ice-cold polythene tubes, centrifuged at 0° C and the plasma removed. Ten volumes of acetone were added to the plasma to precipitate the proteins. At this stage the supernatant was stored overnight in the deep-freeze and extraction continued next morning. The supernatant was reduced by vacuum distillation to remove the acetone. The residue was washed twice with three volumes of ether, which was removed by suction. Then the ether residues were blown off at 37° C with nitrogen, and the extracts were then assayed at pH 7.4. Polythene apparatus was used to prevent the possible formation of plasma kinin which may act as an interfering oxytocic principle.

Method of Assay

Extracts were assayed for oxytocic activity against Pitocin (Parke, Davis and Co.) and at times against the synthetic oxytocin, Syntocinon (Sandoz Ltd.), using the superfused rat

Received December 26, 1960.

TABLE 1. OXYTOCIC ACTIVITY OF ACETONE EXTRACTS OF HUMAN FEMALE BLOOD

	Non-pregnant (winter) (*n=13)			†Pregnant (winter) (n=15)			Primiparous (n=16)			Multiparous (n=26)		
	Oxytocic activity	Oxytocin	Oxytocic substance	Oxytocic activity	Oxytocin	Oxytocic substance	Oxytocic activity	Oxytocin	Oxytocic substance	Oxytocic activity	Oxytocin	Oxytocic substance
Mean (mv. /ml. Blood)	0.42	0.12	0.38	2.89	1.17	1.72	2.04	1.79	1.76	2.51	0.79	1.68
S.D.	0.33	0.13	0.35	3.33	1.84	1.73	2.57	2.62	1.35	3.50	1.00	1.82
Fiducial Limits (p=0.05)	0.22–0.62	0.02–0.22	0.10–0.66	2.68–3.10	0.14–2.20	0.76–2.69	0.68–3.40	0–5.04	0.09–3.43	1.09–3.93	0.12–1.46	0.37–2.99

* n = the number of observations.
† Includes 13 women in 3rd Trimester, 3 in 2nd Trimester.

uterus technique as described by Gaddum (13). In most cases the extracts were assayed in parallel on both the superfused uterus and the uterus in the classic organ bath as described by Holton (14), and Gaddum, Peart and Vogt (15). When the extracts were treated with sodium thioglycollate (16, 17), "residual" oxytocic activity could be demonstrated in every instance. This residual activity could be attributed to an unidentified principle which will be referred to as oxytocic substance (O.S.). The possibility that O.S. was 5-hydroxytryptamine or acetylcholine could be excluded by adding Dibenamine hydrochloride and atropine to the perfusing fluid or organ bath. Nor could the residual oxytocic activity be attributed to potassium which was present in the test doses in sub-threshold concentration as verified by flame photometry.

RESULTS

Reference to Table 1 shows that acetone extracts of blood from healthy non-pregnant medical students possess oxytocic activity which is due to oxytocin and to oxytocic substance. These blood samples were collected during the latter part of the winter. Table 2 shows the mean O.A. of extracts of blood taken from the same non-pregnant women during spring. The mean O.A. of the blood obtained during winter was significantly greater (P = 0.005) than that of the corresponding samples obtained in spring.

Extracts of blood taken during the spring from pregnant women in the first trimester were found to contain significantly greater O.A. (P = 0.01) than that of the spring non-pregnant control group (see Table 2).

In the same table, it may be seen that as pregnancy advances into the second trimester, the mean O.A. of the blood extracts increases and is significantly greater (P = 0.02) than that found in the first trimester.

During the third trimester, however, the mean O.A. did not differ significantly from that obtained during the second trimester. It should be borne in mind that the first and second trimester patients were examined in the spring, whereas those in their third trimester were studied in winter.

Despite the fact that the mean value for O.A. in non-pregnancy was so much greater in the winter than in spring, the mean O.A. in the first trimester of pregnancy (spring) was still significantly larger (P = 0.04) than that in the winter non-pregnancy group.

TABLE 2. OXYTOCIC ACTIVITY OF ACETONE EXTRACTS OF HUMAN FEMALE BLOOD

	Non-pregnant (spring) (*n = 16)	Pregnant (spring) 1st trimester (n = 10)	Pregnant (spring) 2nd trimester (n = 14)	Pregnant (winter) 3rd trimester (n = 18)
Mean (mv./ml. of Blood)	0.12	0.63	3.07	2.70
S.D.	0.06	0.65	3.58	3.37
Fiducial Limits (p = 0.05)	0.086–0.154	0.17–1.09	1.0–5.14	1.03–4.37

* n = the number of observations.

Included in Table 1 are the mean values for O.A., oxytocin and O.S. in the blood of 16 pregnant women all of whom were in the last trimester with the exception of three. The means for O.A., oxytocin and O.S. are significantly greater ($P=0.009$, $P=0.025$, $P=0.006$ respectively) in pregnancy than in non-pregnancy. Both groups were studied in the winter.

Lastly, in the primiparous and multiparous women investigated (see also Table 1), there was no significant difference in O.A., oxytocin or O.S. content of the blood extracts examined.

DISCUSSION

The O.A. of the acetone extracts of blood was higher in pregnant women than in non-pregnant women and so confirms an observation previously reported by Hawker and Robertson (1). The concentration of oxytocin and O. S. increases early in pregnancy and continues to rise up to about mid-pregnancy. From here until near term the concentration of each remains at a high level. These high values for oxytocin are not in accord with observations of other authors (18, 19, 20) who postulate much lower oxytocin values during pregnancy. The high level of oxytocin in blood during pregnancy is an interesting observation and we are not able to explain its significance in view of the relative quiescence of the uterus during pregnancy. Others have reported on the change in the concentration of oxytocinase (18, 21, 22), the half-life of oxytocin (18, 23, 24, 25, 26), and the sensitivity of the uterus to oxytocin (20) during pregnancy. These various observations no doubt are all implicated but it is not possible to show their integration at this juncture.

It has previously been shown that a substance closely resembling and probably identical with vasopressin exists in higher concentration in the blood of pregnant than of non-pregnant women (27, 28). It would therefore appear that during pregnancy the blood concentration of both posterior pituitary hormones is raised.

Finally, it appears that there may be some seasonal variation in oxytocic activity in the non-pregnant woman. The significance of this finding is not immediately apparent, but may in some way be connected with electrolyte balance as suggested by certain authors (29, 30).

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Acknowledgment

We wish to thank the National Health and Medical Research Council (Australia) for a grant to one of us (R.W.H.).

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FAILURE TO CONFIRM THE REPORT THAT PITRESSIN HAS A GROWTH STIMULATING EFFECT[1]

ABSTRACT

Pitressin injected once daily in doses of 0.5 or 2.0 units has no effect on growth in young male rats as measured by body weight, tibial length or tibial epiphysial width. Previous reports of somatotrophin releasing effects of Pitressin could not be confirmed.

Del Vecchio *et al.* (1) have reported that daily injections of Pitressin induced an increase in tibial epiphysial width in young rats, a result interpreted by them as being due to accelerated somatotrophin release. Similarly, Hiroshige and Itoh (2) have reported that Pitressin, gives a tibial epiphysial widening effect and also increases liver RNA concentration in the rat. Some question about the significance of these results has been raised in our minds for two principal reasons. First, the duration of action of Pitressin after intraperitoneal injection, as measured by its antidiuretic effect, is very brief (less

Received December 27, 1960.

[1] This research was supported in part by U. S. Public Health Service Grant B834.

than 1 hour in water-loaded rats). Second, studies carried out in this laboratory indicated that the growth impairment observed in rats with massive hypothalamic lesions, though accompanied by diabetes insipidus was not ameliorated by hormone replacement treatment which included Pitressin tannate injections sufficient to restore external water balance to normal (3). The original experiment of del Vecchio *et al.* (1) was therefore repeated exactly as performed by these authors. In addition, two other series of studies were made using different sized animals and larger doses of Pitressin. Other measures of growth, i.e., body weight change and tibial length were also recorded.

METHODS

Young male Holtzman rats fed Purina chow and water ad libitum and housed in groups in a controlled temperature room (26 ± 1° C) were injected once daily intraperitoneally with Pitressin (Parke, Davis & Company) in a dosage indicated in Table 1. Control animals were injected with 0.5 ml. of physiological saline. At the end of the tenth day the rats were killed

TABLE 1. EFFECT OF PITRESSIN INJECTIONS ON BODY WEIGHT, TIBIAL LENGTH AND TIBIAL EPIPHYSIAL WIDTH IN THE RAT

Group	No. of animals	Body wt.		Tibial length		Tibial epiphysial width	
		Initial (gm.)	Final (gm.)	cm.	±S.E.	micra.	±S.E.
I. a) 0.5 u. Pitressin/day × 10	11	49	89	2.60	.02	367	6
b) control	11	49	91	2.64	.01	368	5
II. a) 2.0 u Pitressin/day × 10	8	125	183	3.28	.04	318	12
b) control	8	127	196	3.28	.01	367	24
III. a) 2.0 u. Pitressin/day × 10	6	131	194	3.29	.03	290	18
b) control	6	133	212	3.33	.03	304	7
*Statistical Analysis (P values)**							
I. a vs. b				< .2		> .9	
II. a vs. b				1		< .1	
III. a vs. b				< .4		< .5	

* Calculated using the method of Snedecor (6).

with ether and the tibiae were removed. Overall length of the bones was measured with a vernier caliper and tibial epiphysial width measured with a micrometer eyepiece after preparation by the method of Evans *et al.* (4). In the experiment repeating the original study of del Vecchio *et al.* (1) the tibial epiphysis was measured by two observers using separate legs. The other measurements were made by a single observer; in one case duplicate examinations of the two legs were made; in the other, only the right leg was used.

RESULTS AND DISCUSSION

As shown in Table 1, no evidence of growth stimulating effect of Pitressin injections was obtained, as measured by change in body weight, lengthening of the tibia or widening of the tibial epiphysis. Reports of STH releasing effects of Pitressin (1, 2) therefore were not confirmed.

Previous studies from this laboratory have indicated that rats with basal hypothalamic lesions of certain size do not grow well (3, 5, 6), that the growth impairment is not ameliorated by replacement with ADH in addition to other endocrine substances (3) and that the growth hormone content of pituitaries from these animals is markedly reduced (6). Although the mechanism of reduced growth following hypothalamic lesions remains obscure, the observation that replacement ADH does not restore growth in brain-lesioned rats and that ADH given to normal rats does not increase growth makes

it highly unlikely that vasopressin is involved in the regulation of secretion of growth hormone.

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CHANGES IN THE SECRETION RATE OF THE RABBIT OVIDUCT FOLLOWING ORAL ADMINISTRATION OF 1-(P-2-DIETHYLAMINOETHOXYPHENYL)-1-PHENYL-2-P-ANISYLETHANOL[1]

ABSTRACT

Using a system for continuous volumetric collection of oviduct secretions, the effect of 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol (MER-25) on the secretion rate of the rabbit oviduct was assessed. After a baseline secretion rate had been established, 225 mg. of MER-25 was administered by mouth daily in 3 divided doses for 3 days to New Zealand White rabbits weighing 3–5 Kg. Intact animals and estrogen primed castrates (4 μg of estradiol 17 β daily) displayed a significantly decreased oviduct secretion rate ($P < 0.05$) during MER-25 administration. The rate reached its lowest level in the 24 hour period immediately following treatment, and increased thereafter to pre-treatment levels. Castrates displayed a significantly increased secretion rate ($P < 0.05$) on the third day of treatment. The rise continued, reaching a peak on the fourth day after the end of treatment, falling thereafter. The changes observed among intact animals and estrogen primed castrates are interpreted as an antiestrogenic effect. The rise in secretion rate in the castrate suggests an estrogenic effect. The possibility of a competitive action at the end organ site between estrogen and MER-25 is suggested.

One-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol (referred to as MER-25), a non steroidal compound with antiestrogenic properties (1), has an antifertility effect in the rat (2) and the rabbit (3). When administered during the first 3 to 4 days of gestation, MER-25 causes degenerative changes in developing ova while still in the oviduct. There is some indication that this effect may be the result of a direct action of MER-25 on the zygote (3, 4). An alternate possibility is that MER-25 may act indirectly by modifying the environment provided for the developing zygote by the oviduct. The present study was designed to explore the effect of MER-25 on the oviduct secretion rate of the rabbit.

Received January 17, 1961.

[1] This work was supported by a grant from the Josiah Macy, Jr. Foundation. The authors are grateful to Mrs. Julia Zalokar, of the Department of Preventive Medicine, Yale Univefsity, for the statistical treatment of the data and to Dr. Dorsy Holtcamp of the W. S. Merrell Company for supplying the compound.

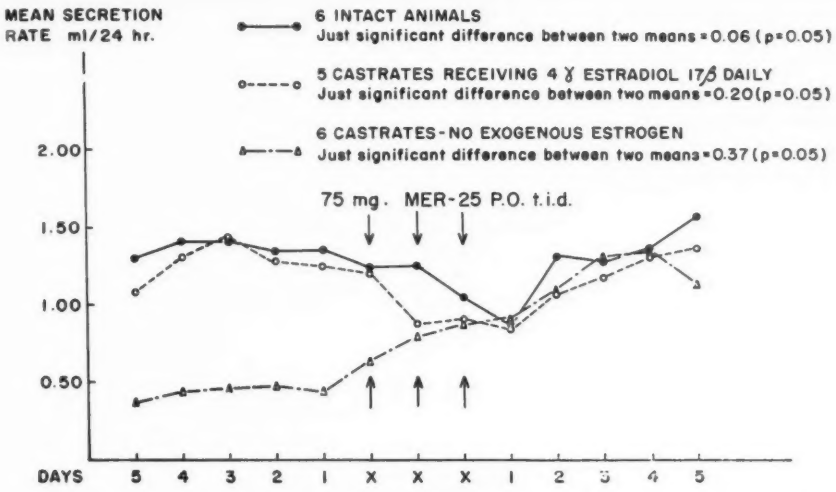
MEAN SECRETION RATE ml/24 hr.
6 INTACT ANIMALS
Just significant difference between two means = 0.06 (p=0.05)
5 CASTRATES RECEIVING 4 γ ESTRADIOL 17β DAILY
Just significant difference between two means = 0.20 (p=0.05)
6 CASTRATES - NO EXOGENOUS ESTROGEN
Just significant difference between two means = 0.37 (p=0.05)
75 mg. MER-25 P.O. t.i.d.
2.00
1.50
1.00
0.50
DAYS 5 4 3 2 1 X X X 1 2 3 4 5

FIG. 1. The secretion rate of the rabbit oviduct (ml./24 hr.) before, during and after oral administration of MER-25.

METHOD AND MATERIALS

A system for prolonged collection of oviduct secretions was applied to New Zealand White rabbits weighing 3 to 5 Kg. Details of the collecting system and the principles underlying its use have been described elsewhere (5). Animals were housed in separate cages and received a diet of Purina chow and water *ad libitum*. After the oviduct secretion rate had been observed over several days to establish a baseline, 225 mg. of MER-25 suspended in olive oil (50 mg./ml.) was administered by mouth daily in three divided doses for three consecutive days. The daily oviduct secretion rate was observed for at least 5 days after treatment in 6 intact animals, 6 castrates and 5 estrogen primed castrates (4 μg of estradiol 17 β S. C. daily).

RESULTS

Among the intact animals there was a significant decrease as determined by analysis of variance ($P < 0.05$) in the mean daily secretion rate on the first day of MER-25 administration (Fig. 1). The mean secretion rate diminished further during treatment, reaching its lowest point in the first 24-hour period after treatment. A return to pretreatment levels followed. An essentially similar effect was observed in estrogen primed castrates (Fig. 1). In this group the decrease in rate became statistically significant on the second day of treatment ($P < 0.05$).

In castrates which did not receive estrogen, the secretion rate increased during MER-25 administration (Fig. 1). The mean daily secretion rate became significantly higher ($P < 0.05$) on the third day of treatment. The rise continued over the succeeding 4 days and fell thereafter. Each animal was subsequently explored to confirm the absence of ovarian tissue.

DISCUSSION

In earlier experiments a marked decrease in the secretion rate of the rabbit oviduct was observed following castration (6). In the castrate the secretion rate increased following estrogen administration. This effect was observed with as little as 0.5 μg of estradiol-17 β. The decreased secretion rate during and after MER-25 administration in intact animals and in estrogen primed castrates can be interpreted as an anti-estrogenic effect. The increase in the secretion rate in the castrate following MER-25 administration

simulates the response of the oviduct to estrogen. This estrogenic effect of an antiestrogenic agent suggests a competitive relationship between the two at the end organ site.

Chang has observed that pronuclear rabbit ova continued to cleave after having been transferred to the oviducts of ovariectomized rabbits (3). It is unlikely therefore that a modification in the secretion rate of the oviduct could of itself affect ovum development. A change in secretion rate might assume importance if accompanied by a qualitative change in the secretions themselves. Investigation of this possibility must await clarification of the physiological role of oviduct secretions in reproduction.

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DIFFERENTIAL EFFECT OF HYDROGEN PEROXIDE UPON THE BIOLOGICAL ACTIVITY OF THYROID STIMULATING HORMONE AND LUTEINIZING HORMONE

ABSTRACT

Oxidation of crude porcine, ovine or bovine LH-contaminated TSH preparations of pituitary origin for one hour at pH 5.5 and room temperature results in an apparent selective inactivation of the LH contaminent. Partially purified ovine TSH preparations behave similarly. In the case of partially purified bovine TSH, however, the decreased LH activity is accompanied by a significant loss of TSH activity.

Attempts to purify and study the physiological role of thyroid stimulating hormone (TSH) have been hampered by the difficulty with which this hormone is freed from its most common hormonal contaminant, luteinizing hormone (LH). Sonenberg and Money (1) have prepared a large number of chemical derivatives of LH-contaminated crude TSH preparations, but were unable to observe a selective inhibition of either hormone. Condliffe, Bates and Fraps (2) have reported the removal of a high proportion of this LH contaminant from TSH preparations by means of the ion exchanger, DEAE-Cellulose. This report describes a simple method for the selective inactivation of the LH-contaminant of TSH preparations, which may be of use to workers interested in the preparation or study of biologically pure thyroid stimulating hormone.

MATERIALS AND METHODS

TSH potency was estimated from the P^{32} uptake of the thyroids of 2 day old chicks according to the method of Lamberg (3). U.S.P. thyrotropin was employed as the TSH standard. LH potency was determined by the ovarian hyperemia method of Ellis (4), except that

Received February 20, 1961.

TABLE 1. THE EFFECT OF pH ON THE SELECTIVITY OF HYDROGEN PEROXIDE OXIDATION

pH of oxidation	TSH			LH		
	Original S.A.* units/mg. protein	S.A. after oxidation units/mg. protein	% original activity remaining	Original S.A. units/mg. protein	S.A. after oxidation units/mg. protein	% original activity remaining
4.0	0.6	0.3	50	0.05	0.008	16
5.5	0.6	0.6	100	0.05	0.004	8
7.0	0.6	0.2	33	0.05	0.030	60
9.5	0.6	0.1	17	0.05	0.030	60

* S.A.—Specific Activity.

1 microcurie of radio-iodinated serum albumin was administered per rat, and the ovaries were allowed to stand in 1N NaOH overnight prior to counting. The LH standard employed was NIH-LH-S1, a gift of the Endocrinology Study Section, National Institutes of Health. Oxidation studies were carried out at room temperature (22–24° C). The reaction was terminated and peroxide removed by immediate dialysis of the oxidized preparation against large volumes of distilled water in the cold (5–8° C) for 48 hours with frequent changes of water. The dialyzed preparations were then lyophilized and stored at room temperature prior to use. The hydrogen peroxide employed was a 30% solution (Baker).

RESULTS

Table 1 outlines the results obtained when 1% solutions of crude bovine pituitary preparations having a TSH potency of 0.6 units/mg. and an LH potency of 0.05 units/mg. were made 1.0M with respect to hydrogen peroxide, and oxidized for a period of one hour at various conditions of pH. At pH 4.0 LH activity is markedly decreased, but there is a concomitant loss of TSH activity. At pH 5.5 there is an apparent selective inactivation of LH with no loss of TSH activity. Similar results have been observed at this pH when oxidation was carried out in 0.1M instead of 1.0M hydrogen peroxide. Oxidation in 1.0M peroxide at pH 7.0 and 9.5 also leads to a marked decrease of both TSH and LH activity. The data suggests that oxidation at pH 5.5 produces the highest degree of specificity with regard to inactivation of LH.

When the crude bovine TSH-LH preparation employed for the experiments outlined in Table 1 was oxidized in 1.0M hydrogen peroxide for three hours, or in 2.0M hydrogen peroxide for one or three hours, loss of both hormonal activities was observed.

Table 2 outlines the results obtained when TSH-LH preparations of varying potencies, derived from porcine, ovine and bovine pituitaries, were oxidized in 1.0M peroxide at pH 5.5 for one hour at room temperature. The TSH potency of the crude porcine material appeared relatively unaffected by the oxidation, while the LH potency

TABLE 2. EFFECT OF HYDROGEN PEROXIDE OXIDATION ON THE TSH AND LH POTENCIES OF PORCINE, OVINE AND BOVINE PREPARATIONS

Source	TSH			LH		
	Original S.A.* units/mg. protein	S.A. after oxidation units/mg. protein	% original activity remaining	Original S.A. units/mg. protein	S.A. after oxidation units/mg. protein	% original activity remaining
Porcine	1.0	0.8	80	0.05	0.009	18
Ovine	1.0	0.7	75	0.08	0.007	9
Ovine	3.7	3.0	81	0.18	0.008	4
Bovine	1.8	0.6	33	0.30	0.020	7
Bovine	0.6	0.6	100	0.05	0.004	8

* S.A.—Specific Activity.

was markedly decreased. The same situation obtained in the case of crude or partially purified ovine preparations. The data indicate, however, that partially purified bovine TSH (1.8 units/mg.) is considerably more susceptible to oxidative inactivation under the conditions employed than is the crude ovine material.

In further studies, oxidation with hydrogen peroxide, under the conditions outlined above, resulted in complete inactivation of ovine and porcine follicle stimulating hormone (FSH).

DISCUSSION

The results presented here are at variance with the report of Albert (5) who found that oxidation resulted in a loss of TSH activity with no loss in gonadotropic activity. It should be pointed out, however, that this worker employed elemental iodine rather than hydrogen peroxide as the oxidizing agent. Although few conclusive deductions can be drawn on the basis of work with crude hormonal preparations, it is interesting to note the varying effect of peroxide oxidation on preparations from different species. Although LH is inactivated to a much greater degree than is TSH in any of the preparations studied, it is apparent that partially purified bovine TSH (1.8 units/mg.) is more susceptible to inactivation than is partially purified ovine TSH (3.7 units/mg.). The observation that the pituitary glycoproteins, FSH and LH, are extremely susceptible to peroxide inactivation, while TSH, which is also a glycoprotein, is not, may also be of significance.

Acknowledgments

It is a pleasure to acknowledge the able technical assistance of Miss Vivian Tate, Mr. William Fugate and Mr. William Newell in this work. Gratitude is also expressed to Dr. A. E. Wilhelmi for a critical reading of the manuscript.

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THE ENDOCRINE SOCIETY

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The American Urological Association offers an annual award of $1000 (first prize of $500, second prize $300, and third prize $200) for essays on the result of some clinical or laboratory research in Urology. Competition is limited to Urologists who have been graduated not more than ten years, and to hospital internes and residents doing clinical or laboratory research work in Urology. Animal research is not necessary.

The first prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Bellevue-Stratford Hotel, Philadelphia, Pennsylvania, May 14–17, 1962.

For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore 1, Maryland. Essays must be in his hands before November 15, 1961.

SECOND ANNUAL MEETING OF THE MEXICAN SOCIETY OF NUTRITION AND ENDOCRINOLOGY

The Second Annual Meeting of the Mexican Society of Nutrition and Endocrinology will be held at San Jose Purua, Michoacan, Mexico, November 24–25, 1961. Short communications, limited to 15 minutes will be presented in Spanish or English. Abstracts not exceeding 350 words should be submitted by August 31st, 1961. Based on these abstracts, papers will be selected for oral presentation.

The full text and figures of the accepted papers will be reproduced and made available to each participant at the beginning of the meetings. Each complete text must therefore be in the hands of the Secretary by October 8, 1961.

Additional information regarding the meeting and hotel accommodations can be obtained from the Secretary: Dr. Carlos Gual, Hospital de Enfermedades de la Nutricion, Calle Dr. Jimenez 261, Mexico 7, D.F.

NEUROENDOCRINOLOGY SYMPOSIUM

The Endocrinology Study Section of the National Institutes of Health is sponsoring a Symposium on Neuroendocrinology to be held on December 6, 7, and 8, 1961, at Miami, Florida. Registration: December 5 (noon to 6:00 P.M.). Opening meeting: December 6, 9:00 A.M. Those interested in attending should address requests for room reservations to:

Neuroendocrinology Symposium
The New Everglades Hotel
Miami, Florida.

By special arrangement single rooms will cost $8.00, rooms with twin beds and double occupancy, $12.00. These rates will also prevail for a period before and after the meeting, and the hotel management should be contacted for details. For information on the symposium and for a program contact A. V. Nalbandov, 102 Animal Genetics, University of Illinois, Urbana, Illinois. Total attendance at the meeting will be limited to about 250 persons and reservations for rooms will be accepted by the hotel on a first-come, first-served basis after May 1.

DECEMBER 6, 7, 8, 1961

THE NEW EVERGLADES HOTEL, MIAMI, FLORIDA

1. Prologue
   Speaker: R. T. Hill
2. Hypothalamic afferents (limbic system, reticular formation, afferents from midbrain, cortical afferents)
   Speaker: W. J. H. Nauta
   Discussant: P. D. MacLean
3. Vascular connections between hypothalamus, posterior and anterior pituitary gland
   Speaker: J. Landsmeer
   Discussant: H. Duvernoy
4. Neurohypophyseal secretions and their origin
   Speaker: W. H. Sawyer
   Discussants: S. W. Smith
   T. F. Leveque
5. Central nervous system and the secretion and release of ACTH
   Speaker: W. F. Ganong
   Discussants: R. H. Egdahl
   E. M. Anderson
6. CNS and the secretion and release of TSH
   Speaker: S. A. D'Angelo
   Discussant: G. W. Harris
7. CNS and the secretion and release of LH and FSH
   Speaker: B. Flerkó
   Discussants: C. A. Barraclough
   G. W. Harris
8. CNS and the secretion and release of prolactin
   Speaker: J. Meites
   Discussants: S. J. Folley
   A. S. Parkes
   H. N. Bruce

9. Physiology of the pituitary gland as affected by transplantation or stalk transection
   Speaker: J. W. Everett
   Discussants: R. K. Meyer
   R. Courrier
   V. Critchlow
   A. V. Nalbandov
10. Recent advances in the chemistry of neuroendocrine chemical mediators originating in the CNS
   Speaker: R. Guillemin
   Discussants: J. C. Porter
   S. D. McCann
11. Role of peripheral nerves in the function of gonads, accessories and duct systems
   Speaker: R. M. Melampy
   Discussants: R. T. Hill
   A. V. Nalbandov
12. Role of light on the neuroendocrine system
   Speaker: V. Critchlow
   Discussant: A. Wolfson
13. Pharmacology of neuroendocrine blocking agents
   Speaker: P. L. Munson
   Discussant: C. H. Sawyer
14. Human neuroendocrinology
   Speaker: C. Lloyd
   Discussants: A. E. Rakoff
   I. A. Mirsky
15. Concluding Session: Synthesis and summary of symposium
   Speaker: R. O. Greep

THE THIRTEENTH ANNUAL POSTGRADUATE ASSEMBLY OF THE ENDOCRINE SOCIETY ENDOCRINOLOGY AND METABOLISM

The Clinical Center, Bethesda, Maryland

October 2–6, 1961

PROGRAM

Chairmen: Dr. Roy Hertz and Dr. F. C. Bartter

Monday Morning, October 2

8:00– 9:00	Registration
	Pituitary and Hypothalamus
	Chairman: Dr. Charles W. Lloyd
9:00– 9:35	Experimental Studies in Neuro-Endocrine Relationships
	Dr. Evelyn Anderson
9:35–10:00	Chemistry of Pituitary Hormones
	Dr. Peter Condliffe
10:10–10:45	Bioassay and Function of Pituitary Hormones
	Dr. Robert Bates
10:45–11:00	Intermission
11:00–11:40	Clinical Disorders of Anterior Pituitary
	Dr. Griff T. Ross
11:40–12:20	Clinical Disorders of Mid-Brain and Posterior Pituitary
	Dr. Charles W. Lloyd
12:20– 1:00	Endocrine Effects of Hypophysectomy and Stalk Section in Man
	Dr. M. B. Lipsett
1:00– 2:00	Lunch

Monday Afternoon

2:00– 5:00	Case Presentations—Panel and Discussion:
	Disorders of Mid-Brain, Posterior Pituitary and Anterior Pituitary
	Drs. Lipsett, Lloyd, Bartter, Ross, McCullagh
5:30– 6:30	Cash Bar Cocktail Party and Reception—Navy Officer's Club

Tuesday Morning

	Parathyroid Disorders
	Chairman: Dr. F. C. Bartter
9:00– 9:30	Metabolic Effects of Parathyroid Hormone
	Dr. F. C. Bartter
9:30–10:00	Hyperparathyroidism
	Dr. F. R. Keating
10:00–10:30	Hypoparathyroidism
	Dr. F. Engel
10:30–10:45	Intermission
10:45–11:15	Osteoporosis
	Dr. Donald Whedon
11:15–11:45	Metabolic Bone Disease (Other than Osteoporosis)
	Dr. F. C. Bartter

11:45–12:15 Calcium Metabolism in Sarcoidosis
Dr. Norman Bell
12:15– 1:00 Panel and Case Presentations
Drs. Bartter, Keating, Engel, Whedon
1:00– 2:00 Lunch

Tuesday Afternoon

Disorders of Carbohydrate Metabolism
Chairman: Dr. E. Perry McCullagh
2:00– 2:30 Carbohydrate Metabolism
Dr. DeWitt Stetten
2:30– 3:00 Insulin—Mechanisms and Assays
Dr. J. Field
3:00– 3:30 Clinical Uses and Mode of Action of Synthetic Hypoglycemic Agents
Dr. F. Lukens
3:30– 5:00 Panel—Cases
Drs. McCullagh, Lukens, Field, Engel

Wednesday Morning

Problems in Reproduction and Gonadal Function
Chairman: Dr. Roy Hertz
9:00– 9:30 The Interpretation of Testicular Biopsies and Sex Chromatin Pattern Tests
Dr. W. O. Nelson
9:30–10:00 Metabolism Effects of Androgenic and Progestational Steroids
Dr. Richard Landau
10:00–10:30 Clinical Syndromes of Testicular Malformation and Dysfunction
Dr. E. P. McCullagh
10:30–10:45 Intermission
10:45–11:30 Sexual Precocity
Dr. Roy Hertz
11:30–12:15 Hirsutism, Stein-Leventhal and Related Syndromes
Dr. M. B. Lipsett
12:15– 1:00 Hormonal Relations between Mother and Fetus in Pregnancy
Dr. Claude Migeon
1:00– 2:00 Lunch

Wednesday Afternoon

Chairman: Dr. Henry Turner
2:00– 2:30 Gonadal Dysgenesis in Phenotypic Females
Dr. Henry Turner
2:30– 3:00 Secondary Amenorrhoea
Dr. Charles W. Lloyd
3:00– 3:00 Fertility and Sterility
Dr. Celso-Ramon Garcia
3:30– 5:00 Panel—Cases
Drs. McCullagh, Turner, Garcia, Migeon, Hertz, Lloyd
6:00 Cocktails (Cash Bar) Dinner for Registrants and Faculty, Navy Officer's Club

Thursday Morning

Thyroid Disorders
Chairman: Dr. Rulon Rawson
9:00– 9:30 Experimental Thyroid Tumors
Dr. S. Wollman

9:30–10:30 THYROID CANCER
Dr. Rulon Rawson
10:30–10:45 INTERMISSION
10:45–11:15 THYROXIN ANALOGUES—THEIR PROPERTIES AND USES
Dr. S. Asper
11:15–12:15 EVALUATION OF MEDICAL THERAPY OF THYROID DISEASE
Dr. F. R. Keating
12:15– 1:00 THYROXIN-BINDING PROTEINS IN IODINE METABOLISM
Dr. J. Robbins
1:00– 2:00 LUNCH

Thursday Afternoon

THYROID (*continued*)
Chairman: Dr. B. Dobyns
2:00– 3:00 INDICATIONS AND PROCEDURES FOR THYROID SURGERY
Dr. B. Dobyns
3:00– 3:30 AUTOIMMUNITY STUDIES IN THYROID DISEASE
Dr. J. Robbins
3:30– 5:00 CASES AND PANEL
Drs. Rawson, Dobyns, Asper, Keating, Robbins

Friday Morning

ADRENAL DISORDERS
Chairman: Dr. F. C. Bartter
9:00– 9:30 ADRENAL PHYSIOLOGY
Dr. Claude Migeon
9:30–10:00 TESTS OF ADRENAL FUNCTION
Dr. B. Kliman
10:00–10:30 ADRENAL INSUFFICIENCY
Dr. J. Beck
10:30–10:45 INTERMISSION
10:45–11:15 HYPERADRENOCORTICISM WITH RESPECT TO HYDROCORTISONE
Dr. F. Engel
11:15–11:45 HYPERADRENOCORTICISM WITH RESPECT TO ALDOSTERONE
Dr. F. C. Bartter
11:45–12:15 HYPERADRENOCORTICISM WITH RESPECT TO ANDROGENS
Dr. Claude Migeon
12:15– 1:00 PANEL AND CASES
Drs. Bell, Engel, Migeon, Kliman, Beck, Bartter
1:00– 2:00 LUNCH

Friday Afternoon

2:00– 3:00 ENDOCRINE ASPECTS OF THE CANCER PROBLEM
Dr. Roy Hertz
3:00– 3:45 LIPID METABOLISM
Dr. D. Fredrickson

FACULTY

EVELYN ANDERSON, M.D., Chief, Section on Endocrinology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

SAMUEL P. ASPER, JR., M.D., The Johns Hopkins Hospital, Baltimore, Md.

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SEYMOUR H. WOLLMAN, PH.D., Laboratory of Physiology, National Cancer Institute, Bethesda, Md.

GENERAL INFORMATION

Registration

8:00–9:00 A.M.—Auditorium, Clinical Center, Building 10, National Institutes of Health.

Registration will be completed in advance by mail. Registrants will simply hand in their receipted registration card as they enter the Auditorium. Late registrants will be accommodated between 8:00 and 9:00 A.M., Monday, October 2.

Fee

Course: $100.00 (Fellows and Residents $30.00)
Daily: $25.00
This fee covers tuition, coffee breaks, Wednesday dinner for registrants and faculty.

Housing

Block reservations are being held until September 15 for course registrants and faculty at the following nearby motels:

Bethesdan Motor Hotel
7740 Wisconsin Avenue
Bethesda, Maryland Tel: OL 6-2100

Intown Motor Hotel
Wisconsin Avenue and Bradley Boulevard
Bethesda, Maryland Tel: OL 4-1400

Individual registrants and faculty members must complete their own arrangements for housing by direct correspondence with these or other nearby motels. Cab service to NIH is available at nominal cost. Housing arrangements are the individual's own responsibility. Reservations should be completed at least 1 month in advance.

Registration for the course should be completed by letter and check to:

Dr. Roy Hertz
Room 12-N-202, Bldg. 10
National Institutes of Health
Bethesda 14, Maryland

Enrollment limited to 100.

PRESENTATION OF CERTIFICATE OF MERIT OF THE ENDOCRINE SOCIETY

Henry H. Turner, The Endocrine Society has thought of you so often for so many years and been so grateful to you that it seems about time to put some tracing of this regard down on formal paper. We celebrate this occasion here tonight. Physician since 1921, you have practiced endocrinology from the difficult days when tools were poor, to the more abundant present when tools are better. Among other contributions, an account of yours in 1938 brought attention to a syndrome which has had incessant study and which now serves to show more of the essentials of man's organization than was thought comprehensible even five years ago. Teacher since 1924 at the University of Oklahoma and wherever else our Society and many others have called on you, you have instructed in the necessary examination of disease. Citizen since birth, you have been a willing participant in the work of the world, both at home in Oklahoma City and outside, wherever affairs have taken you. Secretary-Treasurer of The Endocrine Society since 1941, by a genius for arrangements you have held us all together. And so we come to this Certificate of Merit of The Endocrine Society. Because you have been a good fellow and enjoyed all of this hard work mightily, we shall, in our thoughts, tie this roll of parchment lightly in a gay ribbon as we wish you well, and add a cheer to all the solemn words.

CHANGE OF PUBLISHERS

Following December 31, 1961, The Endocrine Society Incorporated will assume publication of ENDOCRINOLOGY and The Journal of CLINICAL ENDOCRINOLOGY and METABOLISM. All matters and correspondence relating to subscriptions, back volumes, reprints, and advertising may be addressed to the Executive Secretary, The Endocrine Society, Inc., 1200 North Walker Street, Oklahoma City 3, Oklahoma, following December 31, 1961.

ERRATUM NOTICE

In the July 1961 issue of ENDOCRINOLOGY the Meigs and Engel article running head should have read "Corticosteroid Metabolism."